A STUDY OF SUPEROXIDE DISMUTASE ACTIVITY AND SUPEROXIDE PRODUCTION IN KIWIFRUIT

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Acknowledgments

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This thesis is the result of one and half years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

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ABBREVIATIONS

$(NH_4)_2SO_4$	ammonium sulphate
ANOVA	analysis of variance
CAT	catalase
Cu-Zn SOD	copper-zinc superoxide dismutase
dH_20	distilled water
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fe SOD	iron superoxide
GSH	glutathione
H_2O_2	hydrogen peroxide
$HO_2^{\cdot}$	hydroperoxyl
IEF	isoelectric focusing
KCN	potassium cyanide
Mn SOD	manganese superoxide dismutase
$MnCl_2$	manganese chloride
$O_2^{\cdot -}$	superoxide anion
$OH^{\cdot}$	hydroxyl radical
PAGE	polyacrylamide gel electrophoresis
PI	isoelectric point
PMSF	phenylmethylsulfonylflouride
PVPP	polyvinyl poly pyrrolidone
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase

ABSTRACT

The activity of superoxide dismutase (SOD) was determined in three kiwifruit (*Actinidia*) species including *A. deliciosa*, *A. chinensis*, and *A. arguta*. Among the species tested, the highest SOD activity was found in crude extracts prepared from fruit tissues of *A. deliciosa*. The highest enzyme activity was localized in seed, followed by locules, core and outer pericarp (OP). SOD activity in crude extract of whole fruit remained stable for at least one month when stored at -20°C.

The effect of synthetic protease inhibitors (PI) on SOD activity was investigated. Supplementing crude kiwifruit extracts with PI improved SOD activity in freshly prepared extracts, and in extracts stored at 4°C, but had no effect on those stored at -20 C. Among the PI used, iodoacetamide (an inhibitor of cysteine proteases, for example, actinidin which is a principal protease found in kiwifruit) and PMSF (an inhibitor of serine proteases), had the most and least influence on SOD activity in crude kiwifruit extracts, respectively.

There was a significant increase in SOD activity in kiwifruit (that were relatively firm) when the fruits were stored at low temperature (4 C). An increase in SOD activity was also correlated with a decrease in fruit firmness.

Staining fruit tissues with nitroblue tetrazolium (NBT) provided evidence for stress-induced superoxide generation in kiwifruit tissues. Taken together, the changes in SOD activity and the capacity for stress-inducible superoxide production in post-harvest kiwifruit suggest that SOD might play a fundamental role in the storage life/ripening of kiwifruit.

CHAPTER I

Introduction

1.1 The Superoxide Theory of Oxygen Toxicity

The atmosphere of planet earth was anaerobic until the advent of water splitting, O_2-evolving photosynthesis. The accumulation of O_2 changed the environment for, and therefore changed the selection pressures on, all living organisms. It also increased the mutation rate and therefore hastened subsequent evolution. Advantages could be gained by using the O_2 to increase the useful energy derivable from foodstuffs, to carry out novel metabolic transformations, to solubilize and detoxify numerous compounds and even to generate heat and light.

But there was a price to pay for these benefits and that was to provide defenses against the considerable toxicity of this paramagnetic gas. Those organisms that succeeded in developing the requisite defenses could reap the benefits,

and they gave rise to the enormous variety of aerobic life forms that are now so evident on earth. Those that could not accommodate the challenge of O_2 toxicity evolved into the sensitive microscopic anaerobes now restricted to those anaerobic niches that remain even on a thoroughly aerobic planet. So, why is O_2 toxic and what sorts of defenses have been evolved to blunt that toxicity?

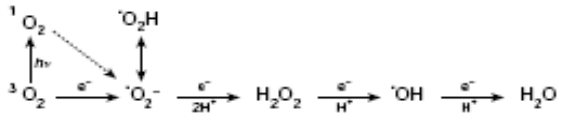
1.1.1 Generation of Toxic Reactive Oxygen Species

Molecular oxygen is produced as a result of the oxidation of water by the photosynthetic electron transport chain. In addition, molecular oxygen is assimilated during photorespiration producing phosphoglycolate. Both of these reactions have positive and negative effects.

The reduction of O_2 to $2H_2O$ requires four electrons. Hence, intermediates may be encountered in this univalent pathway and these are superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). For the purpose of this thesis, the term reactive oxygen species (ROS) is used to describe the products of the sequential of molecular oxygen (Figure 1).

The first step in O_2 reduction produces relatively short-lived ROS that are not readily diffusible: hydroperoxyl ($HO_2\cdot$) and superoxide ($O_2^{\cdot -}$). The half-life for $O_2^{\cdot -}$ is approximately 2–4 μs (Knox and Dodge, 1985; Sutherland, 1991; Smirnoff, 1993). These oxygen radicals are highly reactive, forming hydroperoxides with enes and dienes (Salin, 1987). Furthermore, specific amino acids, such as histidine, methionine, and tryptophan can be oxidized by $O_2^{\cdot -}$ (Knox and Dodge, 1985). In the cellular environment, $O_2^{\cdot -}$ will also cause lipid peroxidation, thereby weakening cell membranes.

(a)



(b)

Superoxide disproportionation

$$2\ ^{\cdot}O_2H \rightleftharpoons H_2O_2 + O_2 \quad (1)$$

$$2\ ^{\cdot}O_2H + {}^{\cdot}O_2^- + H^+ \rightleftharpoons H_2O_2 + O_2$$

$$2\ ^{\cdot}O_2^- + 2\,H^+ \rightleftharpoons H_2O_2 + O_2$$

Catalase

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \quad (2)$$

Peroxidase

$$\text{Peroxidase } (Fe^{3+}) + H_2O_2 \longrightarrow \text{Compound I} \quad (3)$$

$$\text{Compound I} + RH_2 \longrightarrow \text{Compound II} + {}^{\cdot}RH$$

$$\text{Compound II} + RH_2 \longrightarrow \text{Peroxidase } (Fe^{3+}) + {}^{\cdot}RH$$

Generation of H_2O_2

$$^{\cdot}RH + O_2 \longrightarrow {}^{\cdot}O_2^- + RH \quad (4)$$

$$\text{Peroxidase} + {}^{\cdot}O_2^- \longrightarrow \text{Compound III}$$

$$\text{Compound III} + RH_2 \longrightarrow \text{Peroxidase } (Fe^{3+}) + {}^{\cdot}RH$$

$$2\ ^{\cdot}O_2^- + 2\,H \rightleftharpoons H_2O_2 + O_2$$

$$RH_2 + {}^{\cdot}O_2^- \longrightarrow {}^{\cdot}RH + H_2O$$

Halliwell–Asada pathway:

(5)

Haber–Weiss reaction:

$$H_2O_2 + {}^{\cdot}O_2^- \longrightarrow {}^{\cdot}OH + OH^- + O_2 \quad (6)$$

Fenton reaction:

$$H_2O_2 + Fe^{2+}(Cu^+) \longrightarrow Fe^{3+}(Cu^{2+}) + {}^{\cdot}OH + OH^- \quad (7)$$

$$^{\cdot}O_2 + Fe^{3+}(Cu^{2+}) \longrightarrow Fe^{2+}(Cu^+) + O_2 \quad (8)$$

Figure 1: (a) Interrelationship between molecular oxygen and the ROS generated in reactions likely to occur in living plant cells and (b) chemical equations depicting major reactions determining the fate and possible interconversions of reactive oxygen species in plants (Wojtaszek. 1997)

The second O_2 reduction generates hydrogen peroxide (H_2O_2), a relatively long-lived molecule (1 μs) that can diffuse some distance from its site of production (Willkenes *et al.*, 1997; Levine *et al.*, 1994). The biological toxicity of H_2O_2 through oxidation of SH groups has long been known and it can be enhanced in the presence of metal catalysts through Haber-Weiss or Fenton-type reactions.

The superoxide radical ($O_2^{\bullet -}$) has been the subject of much research in recent years due to its role as an agent of oxygen toxicity to cells (Candenas, 1989). Oxygen radicals are byproducts of many biological oxidations occurring in many subcellular locations (Fridovich, 1995). $O_2^{\bullet -}$ is produced at any location where an electron transport chain is present, and hence O_2 activation may occur in different compartments of the cell (Elstner, 1991), including mitochondria, chloroplasts, microsomes, glyoxysomes, peroxysomes, aploplast and the cytosol. While all compartments of the cell are possible sites for $O_2^{\bullet -}$ formation, chloroplast, mitochondria and peroxysomes are thought to be the most important generators of ROS (Fridovich, 1986).

Many conditions that limit productivity, including ozone exposure, metal toxicity, exposure to radiation, wounding, chilling, drought, salinity, heat stress, pathogens, and senescence, result in the enhanced production of ROS (del Rio *et al.*, 2002; Quartacci *et al*., 2001; Hernandez *et al*., 2001).

Reactive oxygen species were reported to cause membrane rigidification, peroxidation of membrane lipids, protein denaturation, and DNA mutation (Borg and Schaich, 1988). They have also been reported to cause leaf senescence, wilting of cut flowers and post-harvest fruit spoilage (Leshem *et al.*, 1986). Increased free radical mediated peroxidase damage and loss of membrane integrity are characteristic of senescing plant tissues (Dhinsda *et*

al., 1981; Thompson, 1984). Senescence in plant tissues is known to be accompanied by changes in membrane permeability. Thus an increase of apparent free space and loss in ability to retain solutes have been demonstrated in ripening of fruits (Sacher, 1973), accelerated ageing of seeds (Parrish and Leopold, 1978) and senescence of green plant tissues (Ferguson and Simon, 1973). These permeability changes during senescence have also been correlated with a simultaneous decline in membrane lipids. Breakdown of cell wall components and membrane disruption lead to cellular decompartmentation and loss of tissue structure (Paliyath and Droillard, 1992).

Free radicals derived from oxygen are largely involved in the senescence process, particularly in membrane deterioration (Dhinsda *et al*., 1981; Droillard *et al.*, 1987), because they induce the peroxidation of membrane lipids, resulting in a loss of membrane integrity and membrane-bound enzyme activities (Bertoli *et al.*, 1996).

Like senescence, fruit ripening is accompanied by deterioration of cell membranes (Sacher, 1973; Ferrie *et al.,* 1994), and the overall process may simply be '… functionally modified protracted form of senescence' (Huber, 1987). Experimental evidence has shown that senescence of various fruits, vegetables and flowers is associated with the degradation of biological membranes, and that loss of membrane integrity is an early and fundamental feature of senescence (Thompson, 1988; Stanley, 1991; Leshem *et al.*, 1992). It has been shown that the formation of superoxide and the accumulation of H_2O_2 increase during fruit ripening as indicated by the accumulation of lipid peroxidation and protein oxidation products (Jimenez *et al.*, 2002; Meir *et al.*, 1991).

It is tempting, therefore, to propose that losses of fruit, vegetable and flower quality, due to environmental stress are the result of membrane degenerative processes characteristic of an accelerated senescence. It has previously been suggested that environmental stress of various types is able to reduce or accelerate many physiological changes in the plant cell that resemble the senescence syndrome (Nooden, 1988).

1.1.2 Plant Defense Mechanisms against Oxygen Toxicity

The levels of ROS inside the cell are maintained at their lowest by the relevant protective mechanisms. However, excesses of reactive oxygen species are produced during particular periods of development as well as in response to various types of stress. Plant cells posses both enzymic and non-enzymic mechanisms which can overcome oxygen toxicity and delay the deleterious effects of free radicals (Figure 2). Among the latter are GSH, cysteine, hydroquinones, mannitol, vitamin C and E, falvonoids, some alkaloids, and beta-carotene (Ames, 1993; Larson, 1998). The enzymic antioxidant defense systems include enzymes capable of removing, neutralizing, or scavenging free radicals and oxyintermediates (Scandalios, 1993). Without these defenses, plants could not efficiently convert solar energy to chemical energy.

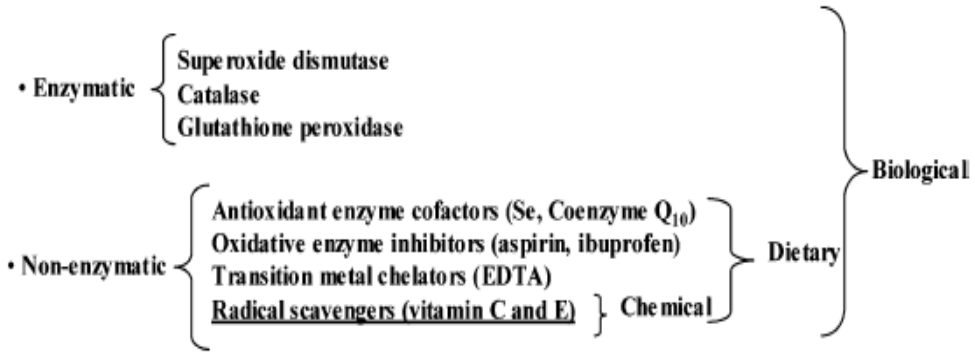


Figure 2: Broad scope of antioxidants (Huang *et al.*, 2005)

1.2 Frontline Defense: Superoxide dismutases

Within a cell, the superoxide dismutases (SODs) constitute the first line of defense against ROS. Superoxide dismutase (SOD, E.C.1.15.1.1) is a critical enzyme responsible for elimination of superoxide radicals and is considered to be a key antioxidant in aerobic cells, because its activity determines the concentration of the Haber-Weiss reaction substrates, $O_2^{\cdot -}$ and H_2O_2 (Khanna-Chopra *et al*., 2004).

SOD enzymes are metalloproteins belonging to a class of oxidoreductase[1] enzymes (Table 1), which catalyse the dismutation of superoxide, the one-electron reduction production product of molecular oxygen,

$$O_2^{\cdot -} + M^{n+1} \rightarrow O_2 + M^{n+} \qquad (1)$$

$$HO_2 + M^{n+} \rightarrow H_2O_2 + M^{n+1} \qquad (2)$$

where M^n is the metalloenzyme in the reduced state and M^{n+1} is the enzyme in the oxidized state to oxygen and hydrogen peroxide (Muscoli *et al.*, 2003).

[1] Oxidoreductases, as the name implies, catalyze the transfer of reducing equivalents from a donor substrate to an acceptor. They are classified on the basis of the nature of the donor and acceptor, with a systematic name consisting of:

"donor:acceptor oxidoreductase"

The subcategories of Enzyme Commission nomenclature for oxidoreductases are based on the functional group of the donor.

Table 1: Examples of some types of oxidoreductases defined in the Enzyme Nomenclature list of the International Union of Biochemistry and Molecular Biology

Oxidoreductases		
E.C. Number	***Oxidoreductase Type***	***Action***
EC 1.1.3.32	Xanthine oxidase	act on the CH-OH group of donors
EC 1.2.1.10	Acetaldehyde dehydrogenase	act on the aldehyde or oxo group of donors
EC 1.6.5.3	NADH dehydrogenase	act on NADH or NADPH
EC 1.9.3.1	Cytochrome c oxidase	act on a heme group of donors
EC 1.11.1.6	Catalase	act on peroxide as an acceptor
EC 1.11.1.5	Cytochrome c peroxiase	
EC 1.11.1.9	Glutathione peroxidase	
EC 1.11.1.7	Myeloperoxidase	
EC 1.14.12	Nitric oxide dioxygenase	act on paired donors with incorporation of molecular oxygen
EC 1.15.1.1	Superoxide dismutase	act on superoxide radicals as acceptors
EC 1.16.3.1	Ceruloplasmin	oxidize metal ions
EC 1.18.6.1	Nitrogenase	act on iron-sulfur proteins as donors

Plants possess three types of SOD with different prosthetic metal groups: Cu/Zn SOD (Cannon *et al.*, 1987; Perl-Treves *et al*., 1988; Bowler et *al.*, 1992), Mn SOD (White and Scandalios, 1988; Bowler et al., 1989), and Fe SOD (van Camp *et al.*, 1991). It has been shown that phospholipid membranes are impermeable to charged $O_2^{\cdot -}$ molecules. Therefore, it is crucial that SODs are present for the removal of superoxide radicals in the compartments where they are formed (Tkahashi and Asada, 1983). Fe SODs are located in the chloroplast, Mn SOD in the mitochondria and the peroxisome, and Cu/Zn SODs in the chloroplast, the cytosol, and possibly the extracellular space (Figure 3).

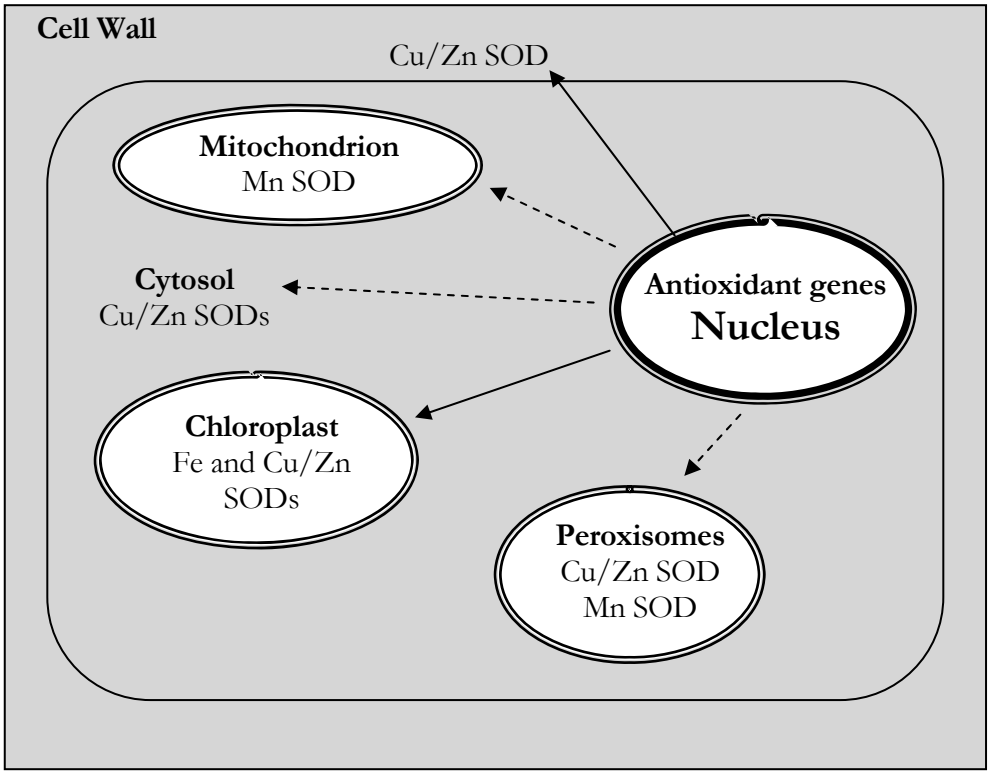


Figure 3: Locations of SODs throughout the plant cell (Alscher *et al.*2002)

1.2.1 Varieties of Superoxide dismutase

1.2.1.1 Fe SODs

The group of Fe SODs probably constitutes the most ancient SOD group. It has been suggested that iron was probably the first metal used as a metal cofactor at the active site of first SOD because of an abundance of iron in soluble Fe (II) form at the time (Bannister *et al.*, 1991). As the levels of O_2 in the environment increased, the mineral components of the environment were oxidized. The decrease in available Fe (II) in the environment caused a shift to the use of more available metal, Mn (III) (Alscher *et al.*, 2002). Fe SOD is inactivated by H_2O_2 and is resistant to KCN inhibition.

There are two distinct groups of Fe SOD. The first group is a homodimer formed from two identical 20 kDa subunit proteins (Yost and Fridovich, 1973). The second Fe SOD group, found in most higher plants, is a tetramer of four equal subunits with a molecular weight of 80-90 kDa (Figure 4B) (Barra *et al.*, 1990).

1.2.1.2 Mn SODs

As mentioned earlier, as the levels of O_2 in the environment increased, the amount of available Fe (II) in the environment decreased, causing a shift to the more available metal, Mn (III). Mn SODs carry only one metal atom per subunit. Mn SOD is either a homodimeric or homotetrameric enzyme with one Mn (III) atom per subunit (Figure 4B). Mn SOD usually has a molecular mass of 40 to 46 kDa, but higher molecular mass Mn SODs have been identified in several species of bacteria with molecular masses of 110 to 140 kDa. The enzyme is not inhibited by potassium cyanide (KCN) or inactivated by H_2O_2

(Alscher *et al.,* 2002). Plant Mn SODs have approximately 65% sequence similarity to one another, and these enzymes also have high similarities to bacterial Mn SODs (Bowler *et al.,* 1994).

1.2.1.3 Cu/Zn SODs

When the atmosphere was completely replenished with oxygen, Fe (II) was almost completely unavailable and insoluble Cu (I) was converted into soluble Cu (II). At this stage, Cu (II) began to be used as the metal cofactor at the active sites of SODs. The electrical properties of Cu/Zn SODs differ greatly from those of Fe and Mn SODs. Therefore, a major change in the structure of the protein occurred after Cu became a metal cofactor (Bannister *et al.,* 1991).

Cu/Zn SODs are found throughout the plant cell. There are two different groups of Cu/Zn SODs. The first group consists of cytoplasmic and periplasmic forms, which are homodimeric. The second group comprises the chloroplastic and extracellular Cu/Zn SODs, which are homotetrameric (Bordo *et al.,* 1994). Cu/Zn SOD is a soluble enzyme, with a molecular mass of 32 kDa and it consists of two identical subunits. Each subunit contains one Cu^{2+} and Zn^{2+} in the active site (Figure 4A).

Cu/Zn SODs are generally very stable enzymes, tolerating exposure to organic solvents and retaining activity in 8.0 M urea (Forman and Fridovich, 1973) or in 2% SDS (Malinowski and Fridovich, 1979). In general the properties and structure of Cu/Zn SOD have been remarkably resistant to evolutionary modifications, and enzymes obtained from plants, fungi, birds and mammals are very similar (Halliwell *et al.,* 1989).

1.3 SOD in Higher Plants and Fruits

Based on the available information, it appears that superoxide dismutases play an important role in protecting plant tissues during different stages of plant growth, development, senescence, and even pre- and post-harvest fruit ripening. SODs protect cells and tissues from damaging effect of superoxide anion by removing it before it reacts with cellular H_2O_2 to form highly reactive hydroxyl radicals ($OH^{\cdot}$), formation of which via the Fenton reaction is favored by excess superoxide (Kumagai *et al.*, 1994; Fridovich, 1978). Cell damage may also be due to the superoxide itself. Therefore, the main function of SODs is to scavenge superoxide radicals generated in various physiological processes, thus preventing the oxidation of biological molecules, either by the radicals themselves, or by their derivatives.

Fruit maturation and ripening are accompanied by alteration in the physical properties of the lipid in membranes and changes in the activity of membrane bound enzymes (Lurie and Ben-Arie, 1983; Thompson *et al*., 1987). Increases in the ion leakage, decreases in the fluidity of plasma membranes, losses of membrane integrity, and accumulation of peroxidised lipids in membranes occur during maturation and ripening of muskmelon (Lester and Stein, 1993), apple (Lurie and Ben-Arie, 1983), and tomato fruit (Palma *et al.*, 1995). It has been reported that the tolerance of plants to stress conditions originating damage may be associated with their higher ability to remove ROS (Senaratna et al., 1985; Ben-Amor et al., 1999) ROS detoxifying enzymes such as SOD may also play a role in protecting fruit from chilling-induced damage during storage at low temperature (Sala, 1998; Sala and Lafuente, 1999). High levels of superoxide dismutase and catalase are involved in delaying the senescence

process. In tomato fruit, SOD and peroxidase activities declined steadily from the immature green stage to the red-ripe stage (Rabinovich *et al.*, 1982).

The importance of SOD in plant response to oxidative damages has been analyzed using transgenic plants overexpressing SOD genes. This approach yielded plants with enhanced resistance to stress conditions (Alscher *et al.*, 2002). Superoxide dismutase is known to be a stable enzyme because of its β-barrel structure and with a low content of α-helix structure (Ursby *et al.*, 1999).

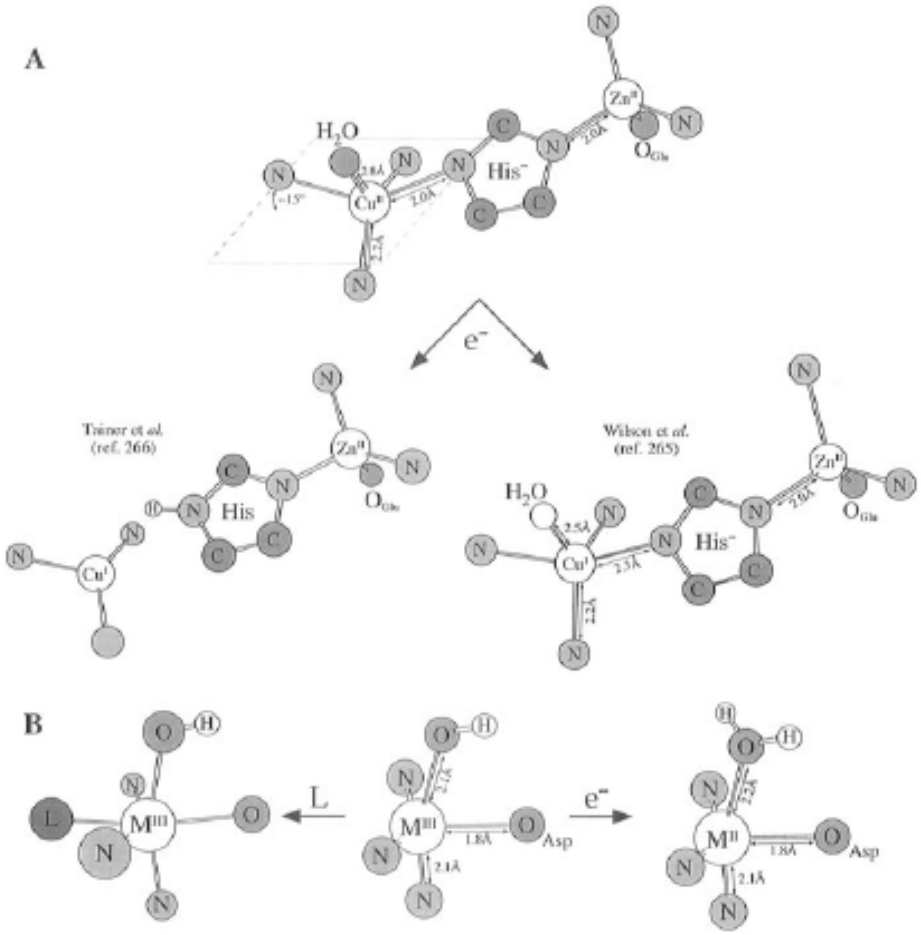


Figure 4: Structural parameters of superoxide dismutases: (A) Cu/Zn SOD in its resting form and two crystallographic forms of the reduced enzyme; (B) Fe and Mn SOD in its resting, ligand-bound, and reduced forms (Holm *et al.*, 1996)

1.4 Kiwifruit

Kiwifruit (Actinidiaceae) is native to eastern Asia. The kiwifruit or Chinese Gooseberry is the fruit of a large woody vine of the genus *Actinidia* (order Ericales, family Actinidiaceae) that grows in temperate climates. It is marketed worldwide as kiwifruit but is more commonly called kiwi in North America. The most common type of kiwifruit, *Actinidia deliciosa*, is about the size of a hen's egg (about 6 cm long and 4.5 to 5.5 cm around), and is often not perfectly round. It has a hairy, dull-brown skin that is not usually consumed. Inside, the flesh is bright green with rows of small, black, edible seeds. The texture of the fruit is soft and the flavor is sometimes described as a mix of strawberry, banana, and pineapple (Science-daily, 2006). The fruit gets its name from the short straight hairs on its skin, which closely resembles the feathers of the kiwi bird of New Zealand. Commercial planting began in New Zealand in the late 1930s and for the past three decades kiwifruit has been increasingly available worldwide. Today, New Zealand is the world leader in the production of quality kiwifruit (Purdue University, 2006).

1.4.1 General Fruit Morphology at Harvest

The fruit, a berry, has a thin epidermal layer, only a few cells deep. The outer pericarp (OP), lying beneath the epidermis, is made up of thin walled elliptical parenchyma cells, separated from the inner pericarp (IP) of elongated cells by a cylindrical network of vascular bundles. Both the outer and inner pericarp cells have plastids containing chlorophyll which give the internal tissue its characteristic green color. Embedded in the inner pericarp is the ring of 20-40 seed locules (carpels), slit like chambers running longitudinally nearly the total length of the fruit. The seed locules contain a mucilaginous matrix which

supports the small black seeds. Within the inner pericarp is the white core, or columella, made up of large parenchyma cells which lack chlorophyll (Beever and Hopkirk, 1990) (Figure 5).

1.4.2 Classification of *Actinidia* spp.

There are 94 recorded species of kiwifruit, of which the following are cultivated for their fruit:

- **Actinidia deliciosa*, formerly called *Actinidia chinensis*; by far the most common in kiwifruit in the marketpalce (Plate 1B)
- **Actinidia chinensis*, similar to *Actinidia deliciosa*; but with green, yellow and even yellow-red flesh (Plate 1A)
- **Actinidia arguta*, kokuwa, tara vine, or hardy kiwi; smaller (10-15 grams) and with green edible skins and green flesh; hardier than *A. deliciosa*
- **Actinidia cordifolia*, hardy kiwi; similar to *A. arguta*
- **Actinidia purpurea*, hardy kiwi; similar to *A. arguta* but red-fleshed
- **Actinidia kolomikta*, kolomikta, arctic beauty, or arctic kiwi; produces very small fruits, 8 grams or smaller; the hardiest species (to about -40°C)
- **Actinidia polygama*, silver vine; produces small fruits

Breeders have also created hybrids by deliberately crossing the preceding species (Biology daily, 2006).

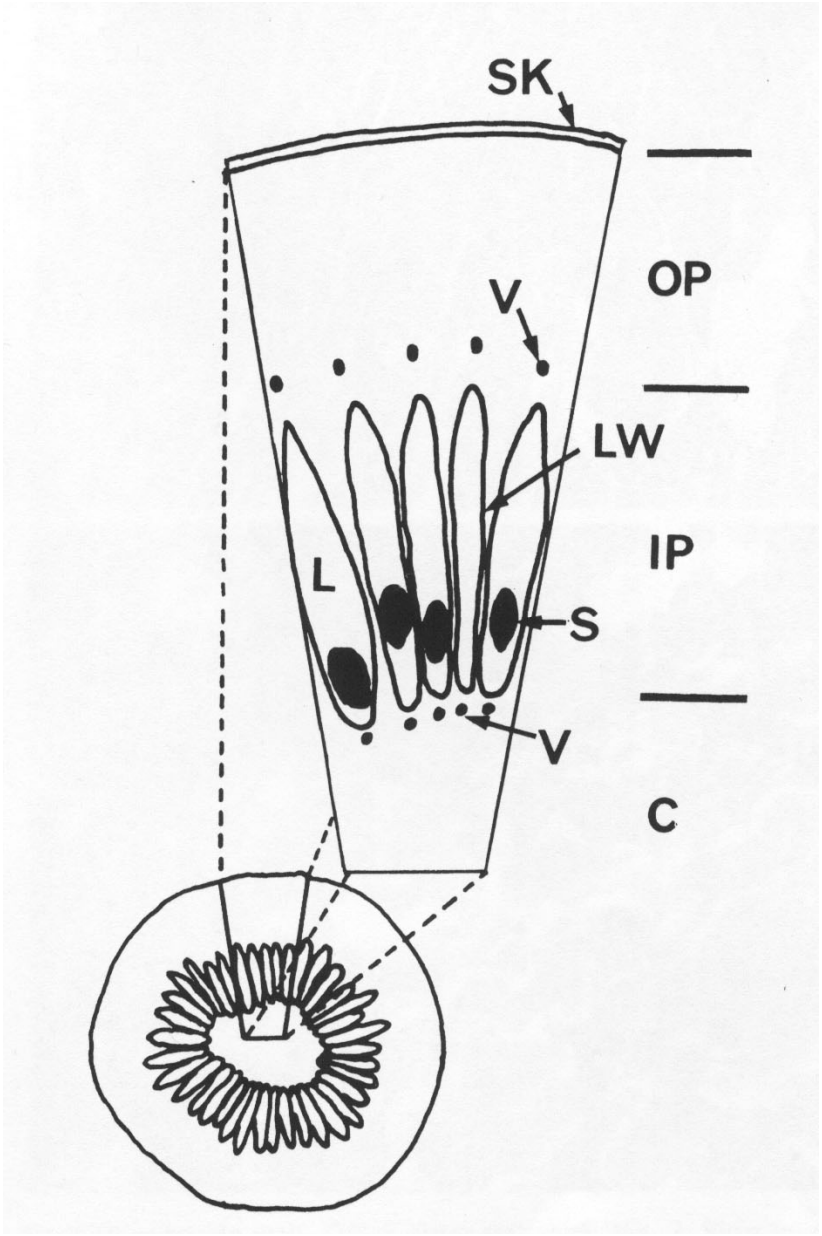


Figure 5: Diagrammatic cross-section of a segment of kiwifruit. *C*= core, *IP*= inner pericarp, *L*= locule, *LW*= locule wall, *OP*= outer pericarp, *S*= seed, *SK*= skin layers (epicarp), *V*= location of major vascular trace (Hallet *et al.*, 1992)

(A) *Actinida chinenesis*

(B) *Actinidia deliciosa*

Plate 1 Two different *Actinidia* species

1.4.3 Fruit Ripening

Kiwifruit shows a long and slow period of growth that lasts for approximately six months from anthesis to until harvest (Gallego et al., 1997). The fruit is very hard while it is developing but firmness decreases slightly during the later stages of development. Once harvested, the initially unripe fruit undergoes dramatic softening (Gallego and Zarra, 1997). The stage of maturity at which any fruit is harvested influences both the ability of that fruit to be stored for long periods, and its final eating quality. This relationship also applies to kiwifruit: if harvested too early, it will never develop full flavor and aroma. Fruits that are physiologically mature but have barely started to ripen can be harvested and will continue to ripen off the vine (Beever and Hopkirk, 1990). Changes in the firmness of fruit tissue involve alteration to the structure of cell walls. There have been reports of changes in the composition and structure of kiwifruit cell walls during the ripening process once the fruits were harvested (Nicolas *et al.*, 1986; Macrae et *al.*, 1989). Physical and chemical parameters of fruit undergo dramatic changes not only in whole fruit but in different tissues of fruit.

1.4.4 Kiwifruit as a Source of Antioxidants

Kiwifruit contains many health beneficial compounds such as vitamin C, carotenoids, flavonoids, minerals, and others (Wills *et al.*, 1986). It is currently accepted that the consumption of fruit-derived antioxidants such as vitamin C, carotenoids, and flavonoids has a preventive effect against certain cancers and cardiovascular disease (Hertog *et al.*, 1993; Miller *et al.*, 1997). Kiwifruit have also been used for the treatment of cancer in Chinese folk medicine (Motohashi *et al.*, 2001). For example, many different cancers of the digestive system (mainly stomach cancer), lung, and liver, have been treated with

kiwifruit prescriptions due to its cytotoxic and antioxidant activities (Collins *et al.,* 1999; Motahashi *et al.,* 2001). It has also been shown that kiwifruit extracts protects against oxidative DNA damage in human cells (Collins *et al.,* 1999).

1.4.5 Acidity of Kiwifruit

The three major acids present in kiwifruit are citrate, quinate, and malate. Kiwifruit contains 0.9-2.5% total acidity with 40-50% as citrate, 40-50% as quinate, and 10% as malate. The citrate and quinate is highest in inner and outer pericarp, respectively. The core has the lowest total acid content; predominantly citrate is present at the time of harvest. The storage temperature affects the balance of the three major acids in the fruit (Richardson *et al.,* 2004).

1.5 Physiology and Applications of Superoxide Dismutase

1.5.1 Physiology

The superoxide anion radical ($O_2^{\cdot -}$) spontaneously dismutes to O_2 and H_2O_2 quite rapidly. However, SOD has the fastest turnover number (reaction rate with its substrate) of any known enzyme. In fact, its rate is diffusion-limited (Peterson et al., 2004). Thus, under real-world intracellular conditions, SOD greatly reduces the ambient level of the dangerous superoxide radical

1.5.2 Applications

The presence of SOD has been shown to help protect many types of cells from the free radical damage that is important in aging, senescence, and ischemic tissue damage. SOD also helps protect cells from DNA damage, lipid

peroxidation, ionizing radiation damage, protein denaturation, and many other forms of progressive cell degradation. SOD is used in cosmetic products to reduce free radical damage to skin, for example to reduce fibrosis following radiation for breast cancer (Compana, 2004).

1.5.3 Quantification of SOD Activity

Principle of Method. Direct quantification of SOD activity has been confounded by the instability of the substrate, superoxide radical. Consequently, quantification of SOD activity often involves an indirect method based on SOD-mediated inhibition of a redox reaction that involves superoxide (Figure 6). The most common form of this assay is based on competition between the reduction on ferricytochrome *c* by the superoxide radical and the SOD-catalyzed dismutation of superoxide. Detector molecules for $O_2^{\cdot -}$ other than cytochrome *c* can also be used, e.g. nitro-blue tetrazloium (NBT) which is reduced by $O_2^{\cdot -}$ to a deep blue colored formazan.

The method used for measuring the SOD activity in this study, was a slight modification of indirect inhibition assay developed by Giannopolitis and Ries (1977), and first described by Beauchamp and Fridovich (1971). In this method, riboflavin was utilized to generate a superoxide flux. Illumination of a riboflavin solution in the presence of EDTA causes a reduction of the flavin. It

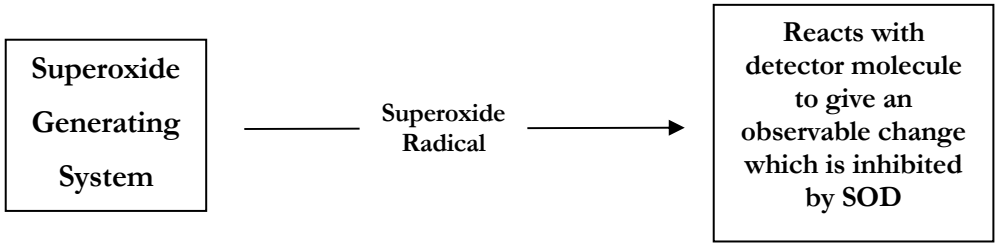


Figure 6: Principle of the indirect assay methods for SOD activity

then reoxidizes and simultaneously reduces oxygen to $O_2^{\cdot -}$, which reacts with a detector molecule such as nitro-blue tetrazloium (NBT). The absorbance obtained from NBT reduction to blue formazan by superoxide was determined spectrophotometrically at 560 nm.

The SOD in the sample competes for superoxide, inhibiting the reaction rate of superoxide with NBT. The percentage of this inhibition is the basis on which the amount of activity is calculated as below:

$$\% \text{ inhibition} = \frac{\text{absorbance (reaction blank)} - \text{absorbance (sample)}}{\text{absorbance (reaction blank)}} \times 100$$

One unit of SOD was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50%.

1.6 Aim and Objectives

The demand for natural products which promote a good health status is growing considerably. Antioxidants are important in human nutrition for their portective role against different pathological situations and the aging processes (Halliwell and Gutteridge, 2000). Besides the low molecular weight antioxidants (ascorbate, glutathione, vitamin A and E, etc.), there are several enzymatic systems, inculding superoxide dismutase which is an important defence line against oxidative stress (Vang *et al.*, 2001; Alscher *et al.*, 2002). Kiwifruit is one of the most consumed and popular fruits, and the knowledge of its nutritional properties, including its antioxidative content is important for human nutrition.

Kiwifruit could be a potential source of SOD with unusual properties. Post-harvest kiwifruit could be exposed to relatively a long period of stress (from low temperature storage to transport handling and dry environment). Also it would be of interset to examine more closely the SOD from a low pH environment that prevails in kiwifruit. The objectives of this study were to:

(i) Determine the SOD activity in kiwifruit extract,

(ii) Study the characteristic of SOD activty in kiwifruit; and

(iii) Examine superoxide production by kiwifruit.

CHAPTER II

Methods and Materials

2.1 Preparation of Extracts

2.1.1 Extraction of SOD Enzyme from Kiwifruit

Ripe mature kiwifruit; A. *deliciosa* ,A. *chinensis* and *A. aguta* fruits used for the purpose of this study, were purchased from a local supermarket from time to time in Christchurch, New Zealand. Although three different species were utilized, unless specified otherwise, most studies were carried out on *A. deliciosa*. Kiwifruit was peeled and cut into small pieces. The tissues were thoroughly ground with a cold mortar and pestle on an ice bath. The grinding medium consisted of 0.1 M of potassium phosphate buffer, pH 7.8, supplemented with 2% (w/v) insoluble polyvinyl polypyrrolidane (PVPP), 0.1 M dithiothreitol (DTT), plus acid washed sands. The weight of tissue (g) to

volume of extraction buffer (ml) was 1:1. The tissues were ground until no fibrous materials were visible. The homogenate was filtered through two layers of synthetic cloth followed by centrifugation for 20 min at 10000×g at 0-4°C. The supernatant was carefully removed, filtered through one layer of synthetic cloth and used as crude SOD extract from whole fruit for determination of SOD activity in fruit tissue.

2.1.2 Extraction of SOD Enzyme from Seeds of Kiwifruit

Ripe fruits were cut into halves, the flesh scooped out and incubated in distilled water for 24 h at room temperature. The pulp was then washed through a sieve, using running dH_2O, leaving the seeds behind. Seeds were then blotted-dry. A known weight of seeds was then ground with homogenizing acid washed sands in a cold mortar and pestle. The resultant powder was homogenized in extraction buffer. The weight of seeds (g) to the volume of extraction buffer (ml) ratio was 1:4. The homogenate was filtered through two layers of synthetic cloth and centrifuged for 20 min at 10000×g at 0-4°C. The supernatant was removed and filtered through one layer of synthetic cloth. The resultant supernatant was used as crude extract of seeds.

2.2 Assay Procedure for Protein

The total protein concentration in crude extracts was measured by using the method of Bradford (1976). Crude extracts were diluted by adding potassium phosphate buffer, pH 7.8, at appropriate ratios. One ml of Bradford reagent was added to 100 μl of diluted crude extract, vortexed and incubated for 5 min at room temperature. Since, the protein-dye complex has a tendency to aggregate with time, therefore in order to do precise determination, the

absorbance of 595 nm, was measured between 5 and 20 min after reagent addition (Bradford, 1976).

2.3 Determination of SOD Activity

As mentioned previously, the ability of enzyme extracts to inhibit light mediated reduction of NBT was the basis for SOD assay. Crude enzyme extracts with appropriate dilution ratio, were added to the reaction mixture consisting of 10 mM EDTA, 63 μM NBT, and 13 μM riboflavin, in a total volume of 1.5 ml potassium phosphate buffer, 0.1 M, pH 7.8. The reaction was started by adding riboflavin, which initiated the light-mediated reaction. Tubes containing the reactants were incubated under fluorescent light, at 22°C. After 25 min, the reaction was stopped by transferring the test tubes to darkness and absorbance of the mixtures at 560 nm was recorded using a BioRad Spectrophotometer. SOD activity was expressed either as enzyme units per gram fresh weight (Ug^{-1} fresh wt.) or units per ml crude extracts. When SOD content was expressed as Ug^{-1} fresh wt., control tubes contained enzyme extracts which were previously boiled for 5 min at 100°C in a water bath, and centrifuged afterwards. For expressing the SOD activity as $U.ml^{-1}$, two sets of identical solutions were prepared. One set of reaction tubes were covered with a black cloth as control, and the other set were illuminated. In either of above measurements, there was no measurable effect of the diffused room light.

2.3.1 Determination of SOD Activity in Crude Extracts of Different Species of *Actinidia*

Crude extracts were prepared from the same weight of A. *deliciosa* and A. *chinensis* as described in 2.1.1. The determination of SOD activity and total protein concentration was carried out as described in 2.3 and 2.2, respectively.

2.3.2 Determination of SOD Activity in Crude Extracts of Different Tissues of Kiwifruit

Each kiwifruit was cut and four different tissues: outer pericarp (OP), locule (L), core (C), and seeds (S) (Figure 5), were separated carefully. Crude extracts from the different tissues were prepared as described in 2.1.1 and 2.1.2. Determination of SOD activity was carried out as described in 2.3.

2.4 The Influence of Kiwifruit Cell Wall Lysis on SOD Content of Fruit Tissues

Fruits of A. *deliciosa* were utilized for this experiment. Fruits of uniform size but with different firmness were selected and divided into two groups: (A) relatively firm, and (B) very soft. SOD activity of different fruit tissues such as OP, IP, and core (Figure 5) were determined in both groups as described in 2.3. Extracts were prepared as described in 2.1.1.

2.5 Determination of Presence of Endogenous Inhibitors and Promoters

Crude extracts were prepared as described in 2.1.1 and 2.1.2, from different parts of the fruit: OP, L, core, and seeds. In order to measure the inhibitor or promoter activity, test tubes containing the reaction buffer plus appropriate volume of crude extract of one tissue, or extracts from two different tissues were pre-incubated at 22°C, for one h. After pre-incubation, substrate was added and SOD activity was determined as described in 2.3. The expected value of enzyme activity for the combined extracts was calculated by adding activities of extracts incubated alone under the same condition. The difference between the expected value and experimental value of the mixed extracts divided by the expected value was expressed as a percentage, indicating the measure of inhibition or promotion.

2.6 Determination of the Effect of Synthetic Protease Inhibitors on SOD Activity

In order to avoid the proteolysis of the enzyme of interest in kiwifruit crude extracts, a protease inhibitor (PI) cocktail containing following solutions:

(i) PMSF, 20 mM,
(ii) Iodoacetamide, 40 mM and,
(iii) 1,10 phenanthroline, 10 mM,

combined with distilled water was prepared, and added to appropriate volume of crude extract, and the resultant mixture was referred to as **PIW**. Accordingly, the same procedure was adopted by combining the inhibitors with potassium phosphate buffer, pH 7.8, to determine the effect of buffer, and

this resultant mixture was referred to as **PIB**. In order to show the influence of each of the above mentioned inhibitors, experiments also were carried out by eliminating one protease inhibitor at a time. Measurement of the SOD activity was calculated as described in 2.3.

2.7 Effect of Storage at Different Temperatures on the Stability of SOD Activity of Crude Extracts of *A. deliciosa*

Enzyme extracts from whole fruit tissue were prepared as described in 2.1.1. Extracts were divided into two groups comprising of (A) extracts supplemented with a cocktail of synthetic protease inhibitors (PI), and (B) extracts without synthetic protease inhibitors. The extracts were stored in Eppendorf tubes as 1 ml aliquots and were kept at room temperature (22°C) for seven days, and at 4°C and -20°C, both for a period of one month. SOD activity and total protein concentration at each case was measured at different time intervals, as described in 2.3 and 2.2 respectively. The activity of the stored samples was compared with that of the freshly prepared extracts.

2.8 Determination of SOD Activity in Fruits Stored at 4°C and 22°C

Post-harvest fruits of *A. deliciosa* were selected based on the uniformity in size and firmness. Relatively firm fruits were used and divided into two groups of (A) and (B), and were maintained in darkness at 22°C, and 4°C, respectively, for the period of 2-14 days. SOD activity and total protein concentration of enzyme extracts (freshly prepared) from each group of fruits was measured at 2-day intervals, as described in 2.3 and 2.2, respectively.

2.9 Characterization of SOD Activity in Extracts of *A. deliciosa*

2.9.1 Thermal Stability

The thermal stability of kiwifruit crude extract SOD was studied by incubating the appropriate volume of crude extracts at 100^{o}C in water bath for different time intervals from 0-20 min. The results were expressed as the percentage of relative activity. SOD activity of extracts was measured as described in 2.3.

2.9.2 Effect of pH

The pH activity profile of SOD in kiwifruit crude extracts was studied at standard assay conditions as described in 2.3, by using three different buffer systems in three different pH ranges. A 0.05 M citrate phosphate buffer was used for the pH range of 2.6-6.0, a 0.1 M potassium phosphate buffer was used for pH 7.2 and 7.8, and Tris-HCl buffer was used for the pH range of 8.5-10. SOD activity was measured as described in 2.3.

2.9.3 Estimation of pH Stability

Appropriate volume of kiwifruit crude extract was added to different buffers having pH values between 2.6 and 10, and pre-incubated for 2 h at 22 $^{\circ}$C. After pre-incubation, SOD activity was measured under a standard assay conditions as described in 2.3.

2.10 Detection of Superoxide Radical by Tetrazolium Salt Staining

Nitro-blue tetrazolium (NBT) was utilized to demonstrate the production and release of superoxide radical in kiwifruit tissues. Fruits were cut into half, and divided into two groups: (A) half fruits were put upside down in Petri dishes containing a solution of 63 μM NBT; (B) half fruits were put upside down in Petri dishes containing a mixture of 63 μM NBT and 10 mM $MnCl_2$. Samples were maintained in darkness at 4°C for 24 h to let the solution soak into tissues. The same procedure was adopted to detect $O_2^{\cdot -}$ anions in different parts of fruit tissue by separating the OP, IP, and core from the whole tissue. Then, samples illuminated by turning the soaked surface of tissues toward fluorescent light. After illumination at 22°C for periods of 2 to 24 h the sites of superoxide generation was noted and photographs were taken.

2.11 Gel Electrophoresis

2.11.1 Native-PAGE

Discontinuous gel electrophoresis of crude kiwifruit extracts was performed. The extracts of whole fruit tissue and seed were prepared as described in 2.1.1 and 2.1.2. Preparation of 12% (w/v) discontinuous polyacrylamide resolving gel and 4% (w/v) stacking gel was based on the instructions of the BioRad Mini protein slab gel manual. The crude extracts of whole fruit and seed were diluted with appropriate volume of kpi buffer (pH 7.8). Appropriate volume of 1 M sucrose was added to diluted crude extracts to increase density and avoid mixing in the reservoir. Five μl of the resultant mixture of each sample were loaded into each well. Generally the electrophoresis steps were run at a

constant voltage (200 V) for 42 min. The resultant gel then soaked in 63 μM NBT solution for 20 min while slowly agitated. Then the gel was transferred to 12 ml of a solution containing riboflavin (0.13 mM), EDTA (10 mM), and phosphate buffer (0.1 M, pH 7.8), and was illuminated overnight.

2.11.2 Mini IEF PAGE

A mini IEF gel electrophoresis apparatus purchased from BioRad (California, USA) was used to detect SOD isoforms in seed or kiwifruit extracts prepared as described in 2.1.1 and 2.1.2. Two μl were applied to each lane. The mini IEF gel was prepared according to the instructions in the manufacturer's instruction manual. The electrophoresis conditions were: 100 volts for 15 min, 200 volts for 15 min and finally 450 volts for one h. After electrophoresis, the gel was treated as described in 2.11.1.

2.12 Purification of SOD by Ammonium Sulphate Precipitation

A known amount of whole kiwifruit (skin excluded) was cut into small pieces and homogenized thoroughly with mortar and pestle in an ice bath. The grinding medium was as described in 2.1.1. The homogenate was filtered through two layers of synthetic cloth, and centrifuged at 10000×g at 0-4 $^{\circ}$C for 20 min. The supernatant was removed and filtered through one layer of synthetic cloth. The resultant supernatant was supplemented by an appropriate volume of a cocktail of PI (prepared with water) as described in 2.6, and incubated at 4 $^{\circ}$C for 2 h, and used as crude extract. The crude extract was brought to 40% saturation with solid ammonium sulphate ($(NH_4)_2SO_4$). After 2 h of stirring at 4 $^{\circ}$C, the precipitate was removed by centrifugation at 10000×g, at 0-4 $^{\circ}$C for 20 min. The resultant supernatant was brought to 60%

saturation with solid ammonium sulphate and stirred for 2 h, then centrifuged at 10000×g, for 20 min at 0-4 °C. The supernatant was decanted and precipitate was dissolved in appropriate volume of 0.1 M potassium phosphate buffer pH 7.8.

The protein suspension was transferred to dialysis tubes and dialyzed against at least two changes of potassium phosphate buffer, pH 7.8, using 100 times of the volume of the sample, and allowed 3-4 h for equilibration while stirring at 4 °C.

2.13 Statistical Analysis

Statistical analysis of the data was performed using STATISTIX 8.0 software. The comparison of the treatments was analyzed using one-way analysis of variance (ANOVA). Where a statistical significance was observed, a Tukey's Honest Significance Difference (HSD) test was performed to determine how significant from the appropriate zero the values were. Standard error were calculated and graphically represented as symmetrical error bars. The presented results are representative of three sample extracts, each of three replicates. The data points are shown as the means with standard errors of triplicates samples.

CHAPTER III

Results and Discussion

3.1 Soluble Protein Determination

There was a significant difference among the fruits of *Actinidia* species (Table 2). *A. deliciosa* showed the highest level of soluble protein content, followed by *A. chinensis* and *A. arguta* (ANOVA, $P<0.05$). Seeds of *A. deliciosa* and *A. chinensis* had higher soluble protein content than whole fruit tissue, on the same fresh weight basis. No significant difference was observed regarding the soluble protein content in seeds of *A. deliciosa* and *A. chinensis* (ANOVA, $P>0.05$).

Table 2. Soluble protein content in fruits and seeds of different *Actinidia* species

Species	Soluble protein content* (mg/ml)
A. deliciosa **(fruit)**	**13.76 ± 0.57**
A. chinensis **(fruit)**	**12.79 ± 0.14**
A. arguta **(fruit)**	**10.32 ± 0.30**
A. deliciosa **(seed)**	**28.76 ± 0.79**
A. chinensis **(seed)**	**28.17 ± 0.62**

* Mean values ± standard errors of data obtained from three different fruit extracts or seed extracts. Three replicate determinations were carried out with each extract

3.2 Establishing Assay for Measuring Superoxide Dismutase (SOD) in Crude Kiwifruit Extracts

It was established that extractable SOD activity was present in the different parts of kiwifruit. The SOD assay was carried out on kiwifruit whole tissue, seed, core, locule, and outer pericarp.

Potassium phosphate buffer (pH 7.8) was used in the grinding medium to extract the SOD from fruits of *Actinidia*. It was found that supplementing the medium with 2% (w/v) insoluble polyvinyl polypyrrolidane (PVPP) and 0.1 M dithiothreitol (DTT) could improve the amount of detectable SOD activity in the crude extracts. Kiwifruit contains more than 80 volatile aroma and flavor compounds including trepenes, esters, aldehydes, alcohols, with varying levels of monotrepenes, and phenolic compounds (Wurms et al., 2003). PVPP was utilized to reduce the possible interference of phenolics on SOD activity. Insoluble PVPP removes the phenolic compounds from the extract (Abbasi et al., 1998). Certain level of increase in SOD activity could be obtained by supplementing the grinding medium with DTT.

Despite modification of the assay with inclusion PVPP and DTT in the extraction buffer, it can not be ruled out that crude kiwifruit extracts still contained compounds that could interfere with SOD assays. The identity and biological significance of these compounds remain to be elucidated. It is plausible, therefore, that interfering compounds that are normally compartmentalized might be released, or created during the enzyme extraction process, and consequently represent artifacts of the assay procedure.

When SOD activity was expressed as $U.g^{-1}$ fresh weight, boiled enzyme extracts were always used as control. After 5 min of boiling at 100°C, enzyme

extracts developed cloudiness which might be due to denaturing of soluble proteins or other organic compounds. Since the absorbance readings of the spectrophotometer could be affected by a cloudy solution, the boiled enzyme extract was centrifuged for 10 min at 10000×g before being added to the assay mixture.

3.3 SOD Activity in Kiwifruit (*Actinidia* spp.)

Three different species of Actinidia comprising *A. deliciosa*, *A. chinensis*, and *A. arguta* were utilized to detect and study the activity of superoxide dismutase in crude extracts of post-harvest kiwifruit. Fruit were purchased from time to time from a local supermarket in Christchurch. Selection criteria were the firmness and size of fruits. It is known that firmness measurement is a good indicator for the texture of kiwifruit (White et al., 2005). However, the exact period for which the fruits had been in storage and/or display was difficult to know. This might explain the natural variation that was encountered among the samples with respect to SOD content. The crude kiwifruit extracts levels of SOD activity was not significantly different among the three species tested. *A. deliciosa* had the highest SOD activity, followed by *A. arguta* and *A. chinensis*. The enzyme activity was expressed as $U.gr^{-1}$ fresh weight (Figure 7). *A. deliciosa* showed the largest variation in SOD activity. The antioxidant activity of food products or beverages may get affected due to conditions of cultivation, collection, genotypes, harvesting time, maturity at harvesting, seasonal variation, cultivars, and storage procedures (Ben-Arie et al., 1982; Kader, 1994; Zafrilla et al., 2003; Marsh et al., 2004). It is known that kiwifruit stored at above 0°C have different metabolism compared to those stored at above 0°C.

3.3.1 SOD Activity in Different Tissues of the Fruit of *Actinidia deliciosa*

Kiwifruit are harvested mature and unripe, and considerable fruit softening must take place before fruits can be eaten. Several studies have described the physiological processes during ripening of kiwifruit after harvest (Nicolas et al., 1986; Arapaia et al., 1987; Lallu et al., 1989). Large changes in a number of physiological and chemical parameters have been recorded not only in whole fruit but also in different tissue types within the fruit. Kiwifruit are made up of three distinct tissue types; outer pericarp (OP), inner pericarp (seed and locule), and core, and these have been shown to differ in chemical composition (Macrae et al., 1989a), rate of softening (Macrae et al., 1989b) and rate of change in cell wall compounds (Redgewell et al., 1992). Here, it was established that SOD activity was found in all tissues at different levels. SOD activity was expressed either as $U.gr^{-1}$ fresh weight or $U.ml^{-1}$ crude extract. In both cases, seed tissue had significantly higher activity (ANOVA, $P<0.05$). However, when SOD activity was expressed as $U.gr^{-1}$ fresh weight, outer pericarp had the lowest enzyme activity (Figure 8). Core showed the lowest SOD activity if the activity was expressed as $U.ml^{-1}$ crude extract (Figure 9). In both cases, significantly higher SOD activity was found in seed tissue, followed by locule. The outer pericarp contained the lowest SOD activity, possibly due to presence of endogenous inhibitors, more acidic condition, and/or higher water content, so the enzyme therein could be highly diluted. Expressing the enzyme activity based on $U.ml^{-1}$ crude extract produced different results, showing the lowest SOD activity in core tissue, which again might be related to water content which is the lowest in that tissue. Moreover, measuring the specific activity ($U.mg^{-1}$ protein) resulted in significantly higher SOD activity for seed, and no significant difference among the three other tissues, despite of slightly lower specific activity for

outer pericarp (Table 2). The specific activity in different parts of the fruit ranges from 17.04 $U.mg^{-1}$ protein to 28.84 $U.mg^{-1}$ protein in seed. Specific activity in seed was not as high as its total activity due to its high protein content. However, seed being a propagule with storage structure had the highest SOD content among the other tissues.

Although, there is a lack of data on the role of antioxidant systems particularly SOD during seed development, ROS generation including $O_2^{\cdot -}$ is known to occur during dehydration of various plant tissues (Smirnoff, 1995) and seeds (Hendry et al., 1992). The ability of seeds to withstand desiccation might be related to their ability to scavenge ROS in order to avoid deleterious events such as lipid peroxidation caused by these compounds (Wolleweher-Ratzer Crawford,1994; Samenza, 1999). These mechanisms would involve enzymes such as superoxide dismutase, catalase and enzymes of ascobate-gelotathione cycle. In non-photosynthetic organs like seed, ROS are generally generated through mitochondrial activity by electron leakage to oxygen (Pantarulo et al., 1988). However, it has been shown that seed germinability might be related to the efficiency of free radical scavenging because this scavenging may affect seed storability and vigor (Priestly, 1986; Baily et al., 1998, 2000).

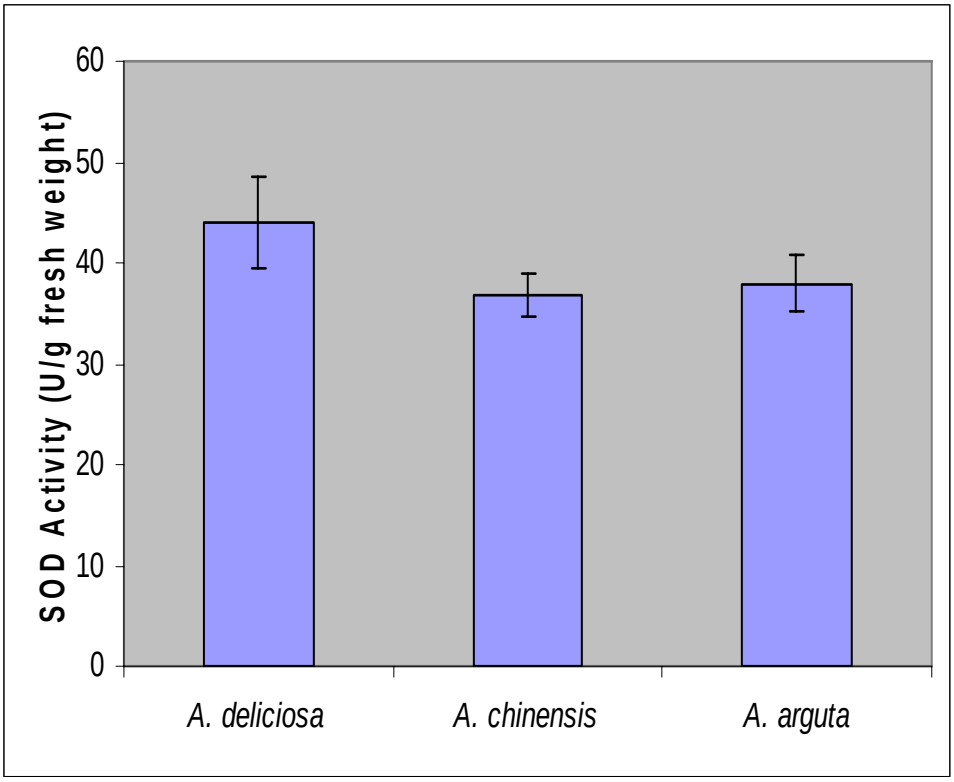


Figure 7. Superoxide dismutase (SOD) activity in fruits of different species of *Actinidia*. SOD activity was determined in three different fruit extracts of *A. chinensis* and *A. arguta*, whereas it was determined in 11 extracts of *A. deliciosa*. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

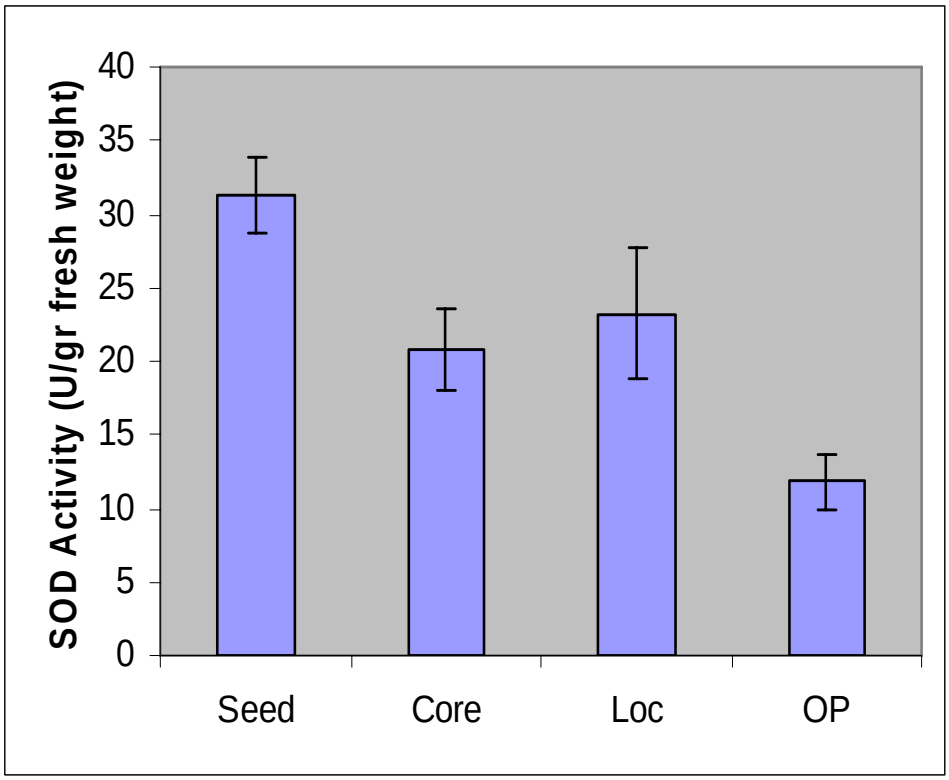


Figure 8. Superoxide dismutase (SOD) activity in different part of the fruit of *A. deliciosa*. SOD activity was determined in three different extracts of each tissue. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

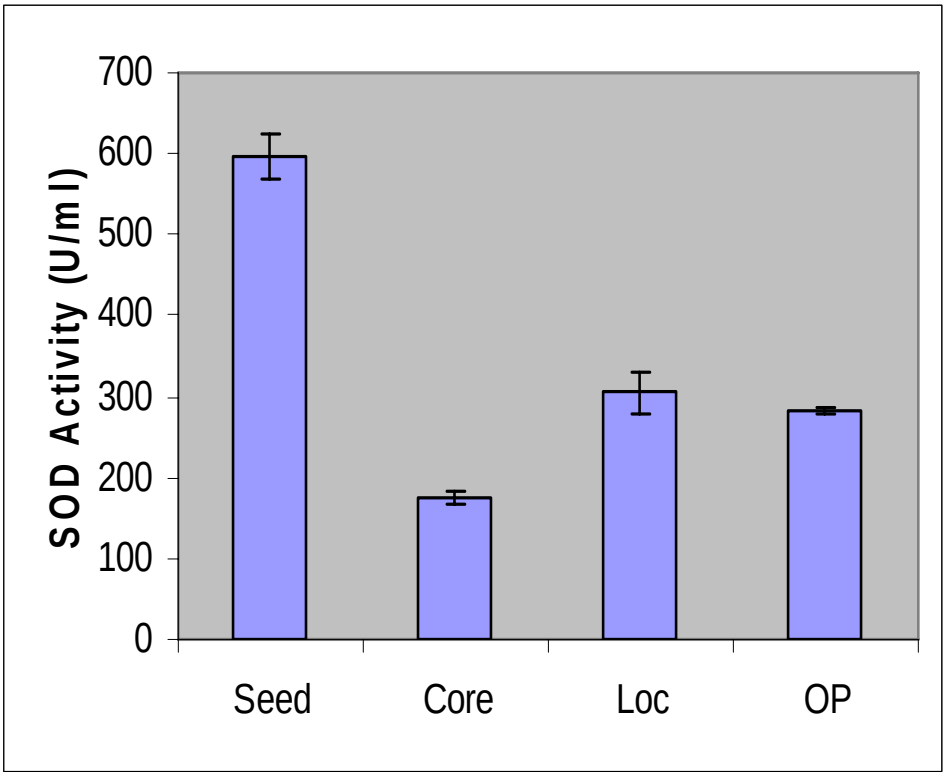


Figure 9. Superoxide dismutase (SOD) activity (u/ml) in different parts of the fruit of *A. deliciosa*. SOD activity was determined in three different extracts of each tissue. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

Table 2. Superoxide dismutase activity in different tissues of *A. deliciosa*

Type of tissue	Specific Activity (U/mg protein)
Seed	**28.84 ± 1.87**
Core	**18.22 ± 0.37**
Locule	**18.57 ± 0.27**
Outer Pericarp	**17.04 ± 0.61**

SOD activity was determined in three different extracts of each tissue. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

3.4 Detection of Localized Accumulation of $O_2^{\cdot-}$ in Fruit Tissues of *Actinidia* Species

Reactive oxygen species (ROS) participate in response to biotic and abiotic stresses (Mittler, 2002). In plant cells, ROS, mainly H_2O_2, superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical are generated in the cytosol, chloroplast, mitochondria, and the apoplastic space (Bowler and Fluhr, 2000; Mittler, 2002). The major ROS scavenging pathways of plants including SOD, found almost in all cellular compartments.

Environmental stress such as drought, excessive light, temperature extremes, air pollution, wounding, or herbicides can disturb normal cellular metabolism. This can upset the balance of oxygen free radical production and quenching. Thus, specific detection of endogenous $O_2^{\cdot-}$ is of critical importance for the study of superoxide production and dismutation. Here, a method was used to detect and demonstrate the localized $O_2^{\cdot-}$ production in kiwifruit tissues by utilizing nitroblue tetrazolium salt (NBT), which reacts with $O_2^{\cdot-}$, producing a blue formazan precipitate. In control samples, $MnCl_2$ which is a highly effective $O_2^{\cdot-}$ dismutating agent was utilized (Hernandez et al., 2001).

Tissues of kiwifruit were shown to respond to wounding by generating superoxide radicals. The magnitude of $O_2^{\cdot-}$ generation was found to be variable between different parts of fruit tissue. Tissues that are more susceptible to stress due to less SOD activity and/or overproduction of $O_2^{\cdot-}$, demonstrated a faster response to the stress by producing a blue formazan precipitation in shorter period of time, which was first detectable in outer pericarp after 2 h of exposure of tissue surface to light (Plate 2). No significant increase in superoxide generation was shown after 4 h, suggesting there might

be an increase in antioxidative activity which could delay the impact of stress (Plate 3). In control samples, no significant sign of blue formazan was found within the first 4 h post-stress, which might be due to inhibitory effect of $MnCl_2$ against superoxide generation. Generation of superoxide reached its greatest magnitude after 24 h (Plate 4), when the blue formazan precipitate was spread over almost whole surface of fruit tissue with greater intensity around more susceptible areas. Blue formazan deposits appeared in parts of tissue in control samples after 24 h (Plate 5), suggesting the loss of inhibitory effect of $MnCl_2$ and/or overproduction of $O_2^{\cdot -}$.

Here, it was shown that cells in fruit tissue exposed to stress, responded with a gradual production of superoxide radical. Susceptible tissues like OP displayed greater intensity of blue formazan, whereas more resistant tissues such as core showed no visible sign of precipitation in initial stage of the experiment – within first 4 h post-wounding. The initial burst of superoxide was probably resulted from plant derived signals. Depending on the type of fruit tissue, the response is proportional to the SOD content (Figure 8). This indicates that, the perception of stress and the signal transduction to the superoxide synthesis complex are related but there is an upper limit defined by other factors such as cell wall strength. Due to the high reactivity of superoxide radical and the diffusion rate in fruit tissue apolplasts, it is likely that the observed results represent the responses of cell layers near the surface of the exposed layer. Cross section of post-stress exposed samples displayed that the radical production approximates more to the surface area rather than the volume of the tissue (Plate 6). When plants are wounded, they form a protective layer next to the exposed surface to prevent dehydration and potential penetration of opportunistic pathogens. This physical barrier termed “suberin” comprises a specific cell wall modification characterized by poly phenolic domain and

wax-embedded poly aliphatic domain (reviewed in Bernards and Lewis, 1998). In potato tubers, wounding triggered the formation of a periderm (i.e. suberized layer) in the tissue immediately below the site of damage (Razem and Bernards, 2003). It is not known if a suberin-like layer would be formed in kiwifruit that has been cut open. Assuming some kind of protective barrier is formed after wounding fruit tissues, the initial deposition of such material in fruit tissue would appear to require at least 10 h, and reached a state in which the 'layer' has sufficient structural integrity to be cut off intact by 20 h post-wounding (Plate 7).

It was also found that when a cut open whole tissue surface of kiwifruit is exposed to oxidative burst, the 'layer' not only act as a barrier for layer underneath, but also could protect the inner tissues from being laterally exposed to wound stress. It was shown that when the core tissue was intact and properly protected by IP and OP, blue formazan was not formed even after 20 h post-stress (Plate 8a). Inner pericarp showed more resistance against oxidative burst, at least until 10 h post-stress, when it was protected by OP (Plate 8a), but once OP was removed, it caused acceleration in deposition of blue formazan in IP (Plate 8b). The core also showed a great magnitude of oxidative burst when it was not surrounded by IP and OP (Plate 8c). It was also demonstrated that isolated IP and OP are more susceptible to oxidative stress than intact tissues (Plate 8d and 8e). Therefore, it can't be ruled out that the oxidative burst in the different tissues at least appeared to correlate with the extent of wounding. This study, also showed that the initial oxidative burst appearing mostly in outer pericarp, might be due to perturbation of cell organization and eventually degradation of enzymes and proteins by released proteases, leading to upset balance between antioxidant enzymes, particularly SOD, and superoxide radicals. Moreover, it was shown earlier that there was

lower SOD activity in OP, which might explain the greater intensity of blue formazan precipitation in this type of tissue. The observation that the second extensive burst appeared in almost whole tissue surface indicates the excessive generation of superoxide radical and/or probably a huge loss of SOD activity due to long-term exposure to stress.

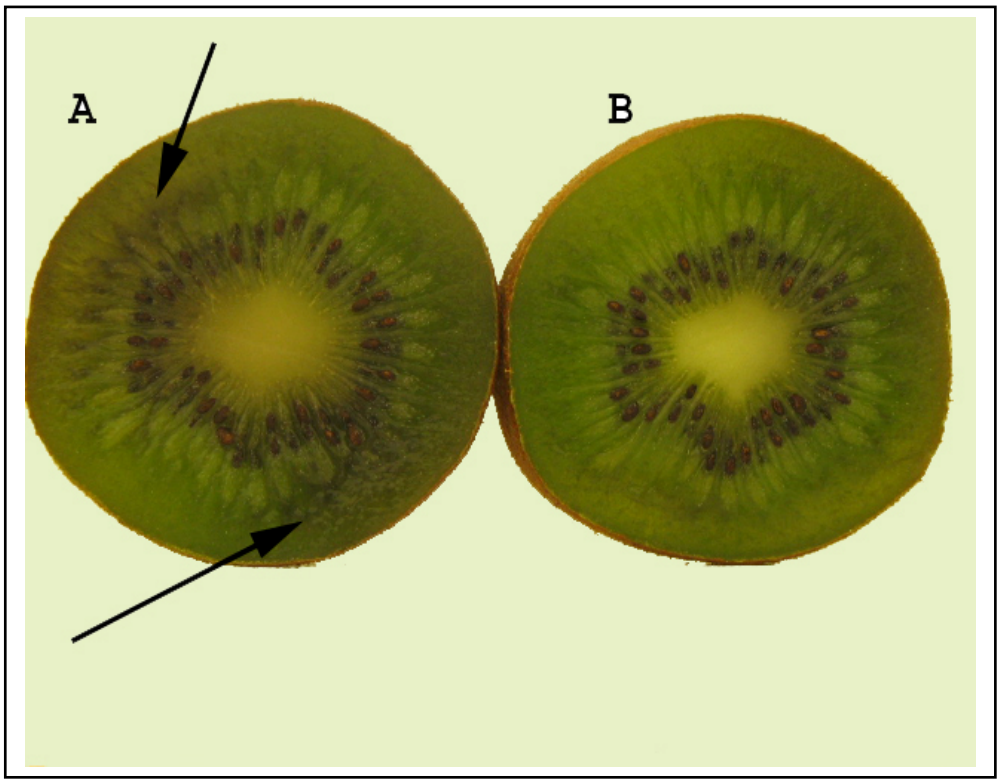


Plate 2 Localized production of superoxide (arrows) in *A. deliciosa* whole fruit tissue after 2 h being exposed to light. Tissue stained with NBT in the absence (A) and in the presence (B) of 10 mM $MnCl_2$.

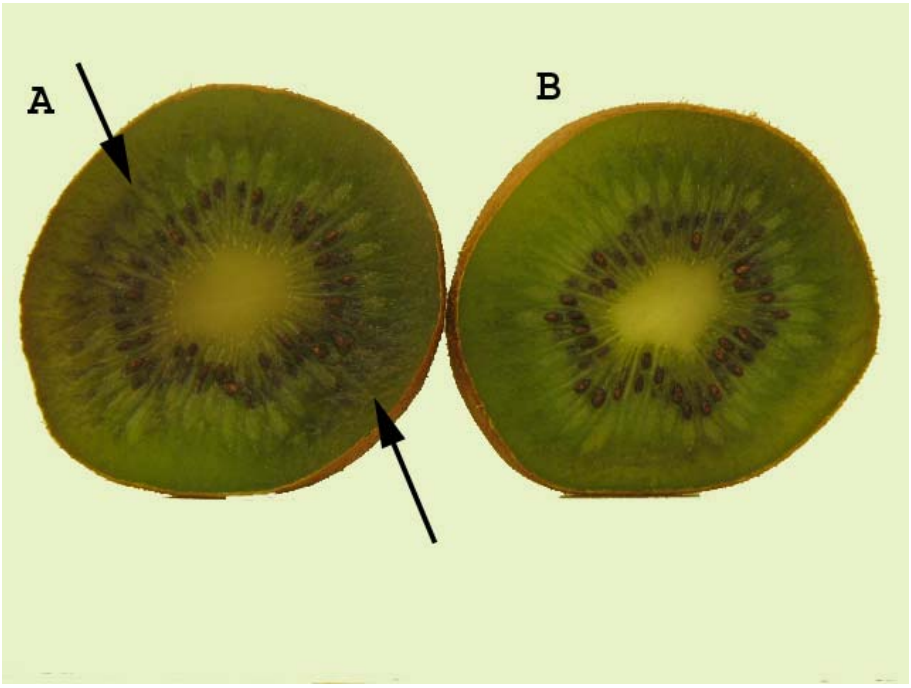


Plate 3 Localized production of superoxide (arrows) in *A. deliciosa* whole fruit tissue after 4 h being exposed to light. Tissue stained with NBT in the absence (A) and in the presence (B) of 10 mM $MnCl_2$.

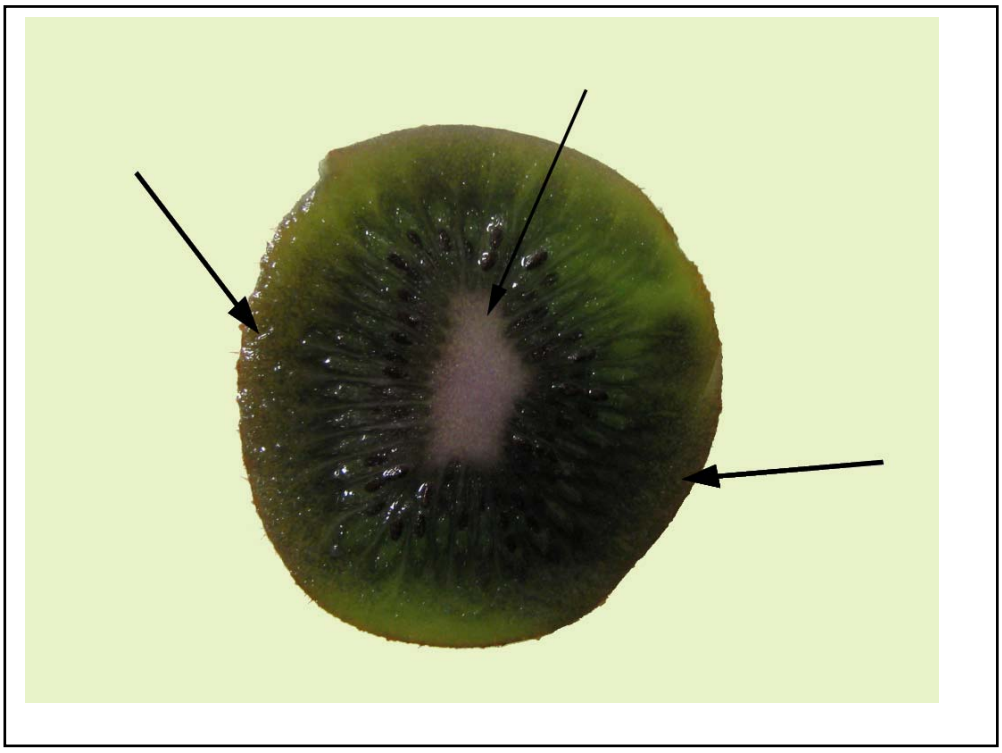


Plate 4 Localized production of superoxide (arrows) in *A. deliciosa* whole fruit tissue after 24 h being exposed to light. Tissue stained with NBT in the absence of 10 mM $MnCl_2$.

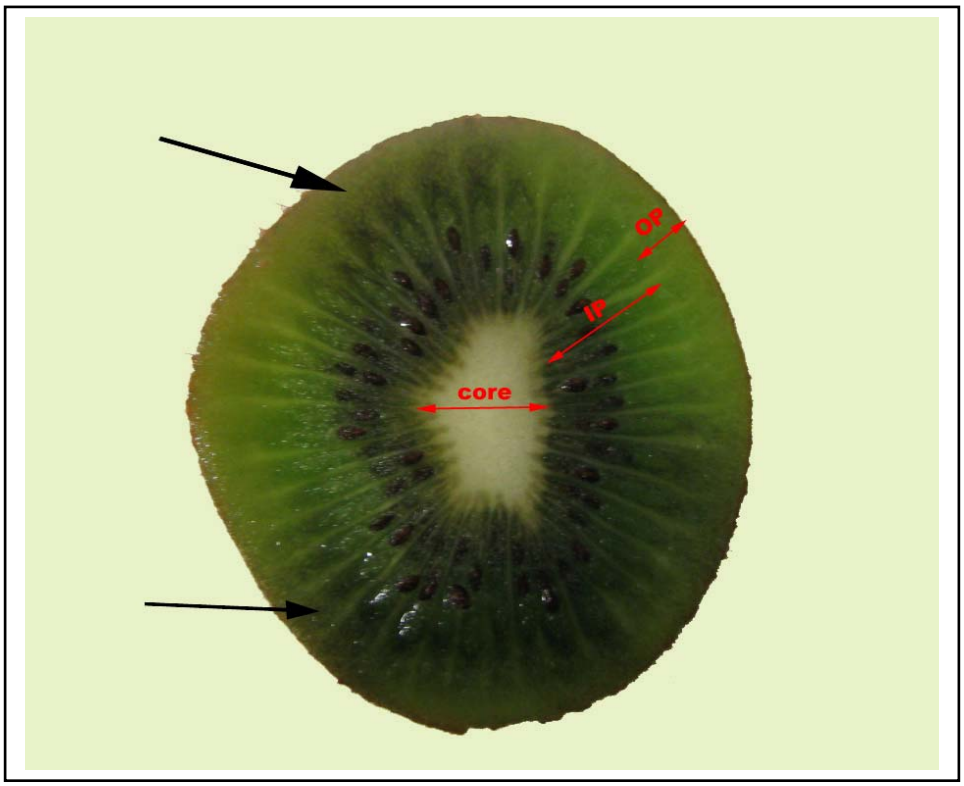


Plate 5 Localized production of superoxide (arrows) in *A. deliciosa* whole fruit tissue after 24 h being exposed to light. Tissue stained with NBT in the presence of 10 mM $MnCl_2$.

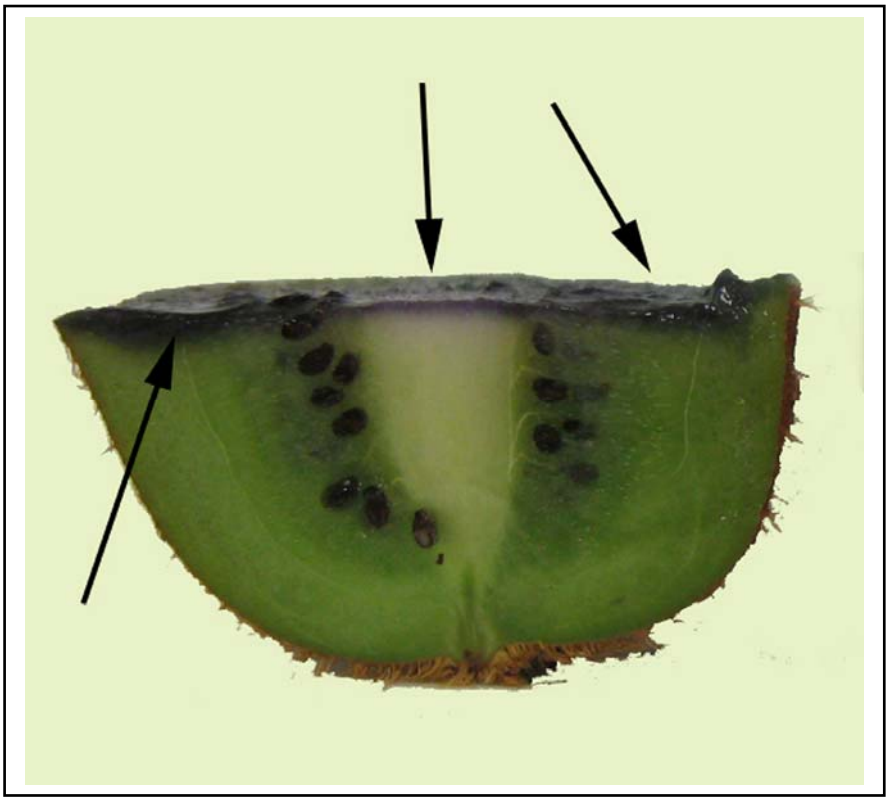


Plate 6 Localized production of superoxide (arrows) on the cut surface of *A. deliciosa* fruit that was in contact with a NBT solution for 24 h. There was no staining in the other cut surface (i.e. the vertical face of the half fruit).

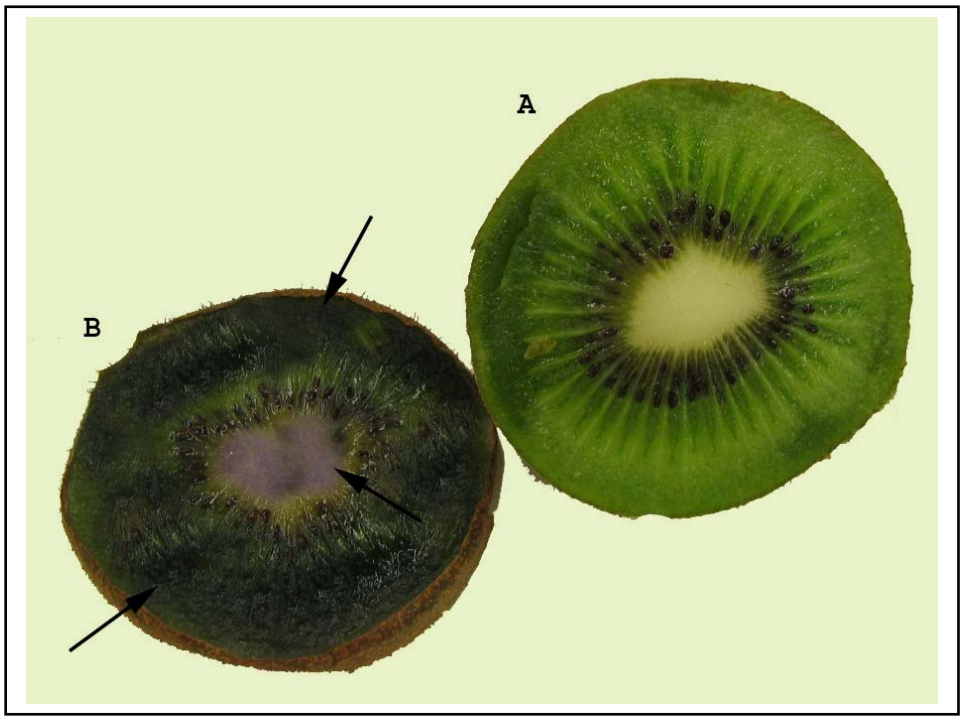


Plate 7 Cross section of exposed tissue of *A. deliciosa* after 20h being illuminated. (A) sample after removing the initial cut-open surface of the fruit, (B) cut-off slice removed from (A). Tissue stained with NBT in the absence of 10 mM $MnCl_2$.

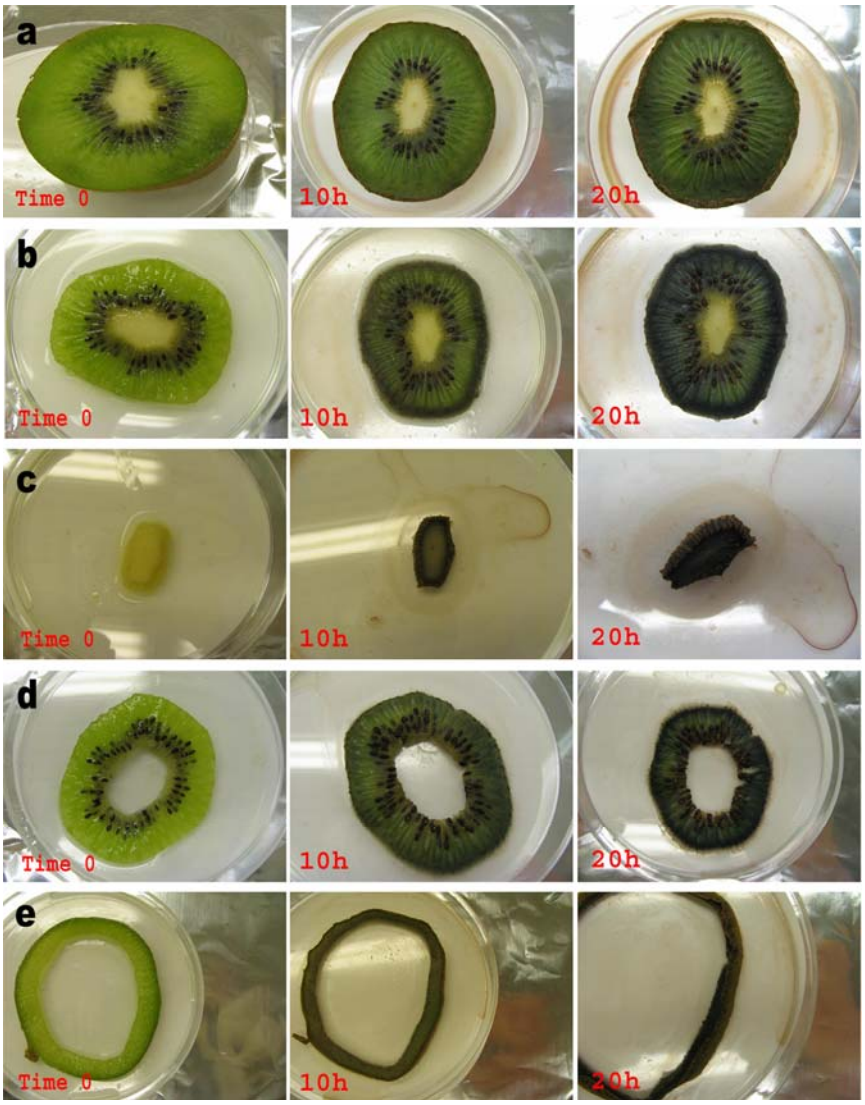


Plate 8 Detection of superoxide in different parts of *A. deliciosa* fruit. (a) cut-open half fruit; (b) fruit slice with the OP removed; (c) isolated core tissue; (d) isolated IP tissue; (e) isolated OP tissue

3.5 Superoxide Dismutase and Its Relationship with the Response of Fruit of *A. deliciosa* to Storage at 4°C and 22°C

Selected fruits of *A. deliciosa* were maintained in darkness at 22°C and 4°C as group (A) and (B) respectively. Measurement of SOD activity was carried out in 2-day intervals for the period of 14 days. Both groups showed no loss of SOD activity at the end of the 14 day storage period (Figure 10). Nevertheless, SOD activity increased significantly in samples of group (B) stored at 4°C, and less significant increase in SOD activity occurred in samples of group (A) stored at 22°C. The highest increase of SOD activity occurred on day 6, as 60% and 100% for group (A) and (B), respectively.

Reactions involving ROS, particularly $O_2^{\cdot -}$, are an intrinsic feature of senescence and fruit ripening. Fruits, including kiwifruit are good source of antioxidants (Vanderslice et al., 1990). It is known that the metabolism of fruit and vegetable crops continues to function despite separation from the plant (Kalt et al., 1999). ROS could be involved potentially in many aspects of storage process. This is important, since specific responses have been observed for different antioxidant enzymes in relation to senescence and ripening (Jimenez et al., 1998; Pastori and del Rio, 1998; Jimenez et al., 2002), which can take place during storage. There are conflicting data concerning changes in SOD activity during ripening and senescence. SOD activity decreased during senescence of leaves (Dhindsa et al., 1981; Jimenez et al., 1998) but increased during senescence of apple fruit (Du and Bramledge, 1994) and potato tuber (Kumar and Knowles, 1993). In Saskatoon fruit, SOD specific activity declined with ripening (Rogiers et al., 1998). SOD activity in tomato fruit declined at beginning of ripening and increased at the over-ripe stage to similar values as in the small green fruits (Jimenez et al., 2002).

To determine the possible association of the oxidative process accompanying the development of fruits of *A. deliciosa* toward ripening and senescence, the pattern of changes in SOD activity of fruits stored at 4°C and 22°C was monitored. It is known that kiwifruit has a long storage life at low temperature. Here, in this study, lack of major changes with respect to softening at 4°C might be due to less major changes in membrane lipids and membrane permeability. Thus, this contributes to maintenance of cellular integrity and possibly to postpone the senescence process in kiwifruit. Results showed a particularly strong increase in SOD activity of fruits stored at 4°C. It has been shown that high level of SOD and CAT contributes to protect potato quality during low-temperature storage (Spychalla and Desborough, 1990). Long storage life muskmelon had high level of SOD and this high level was maintained during post-climacteric stage (Lacan and Baccou, 1997). Observations of the present study also indicate that SOD activity contributes at least to some extent to delay the senescence process in kiwifruit. Obviously, it is not possible to rule out the involvement of other enzymatic and non-enzymatic antioxidants in this process, which were not investigated in this study.

It is known that, fruit ripening is accompanied by the deterioration of cell membrane (Ferrie et al., 1994), pigment accumulation and change in the cell walls that result in softening. These changes were observed as change in flesh color and in the firmness of fruits stored at 22°C (general preliminary observations). It was reported that ROS mediate the induction of genes involved in carotenoid synthesis, and in the transformation of chloroplast to chromoplast (Bouvier et al., 1998). During the senescence process a large increase in generation of ROS, mainly $O_2^{\cdot -}$ and H_2O_2, has been described in different cell compartments, including mitochondria and proxisomes, and such

generation result in a dramatic increase in lipid peroxidation and membrane leakiness (Jimenez et al., 1998; del Rio et al., 1998). Loss of cellular membrane integrity was reported for microsomal membranes during the senescence of bell pepper (Lurrie and Ben-Yoshua, 1986), and the ripening of tomato fruit (Palma et al., 1995). Here, this could mean that fruits in both storage conditions - at 4°C and 22°C – underwent ripening, besides, fruits stored at 22°C were also senescing during storage.

SOD seems to be involved in kiwifruit ripening and senescing during storage, since in both storage conditions, SOD activity increased and reached its peak after 6 d of storage, and then remained almost at steady high level in fruits kept at 4°C, but started declining in fruits maintained at 22°C. Cold stress increased both enzymatic antioxidative activities (SOD and CAT), and α-tocopherol levels thus protecting membrane's polyunsaturated lipids in potato tubers stored at 4°C (Reverberi et al., 2001). Moreover, the content of glucose and fructose increases in response to cold stress (cold sweetening), when potato tubers are stored at below 10°C (Coffin et al., 1987). The low temperature storage induced an increase in the degree of unsaturation and a decrease in the ratio of saturated/unsaturated fatty acids of membrane polar lipids with a subsequent increase of lipid hydroperoxides in potato tubers (Reverberi et al.,2001). Here, results also displayed that concurrently, SOD activity increased with the decreasing of fruit firmness. This indicates that superoxide dismutase was probably an induced enzyme but not the key specific enzyme necessary for the initiation of softening of kiwifruit.

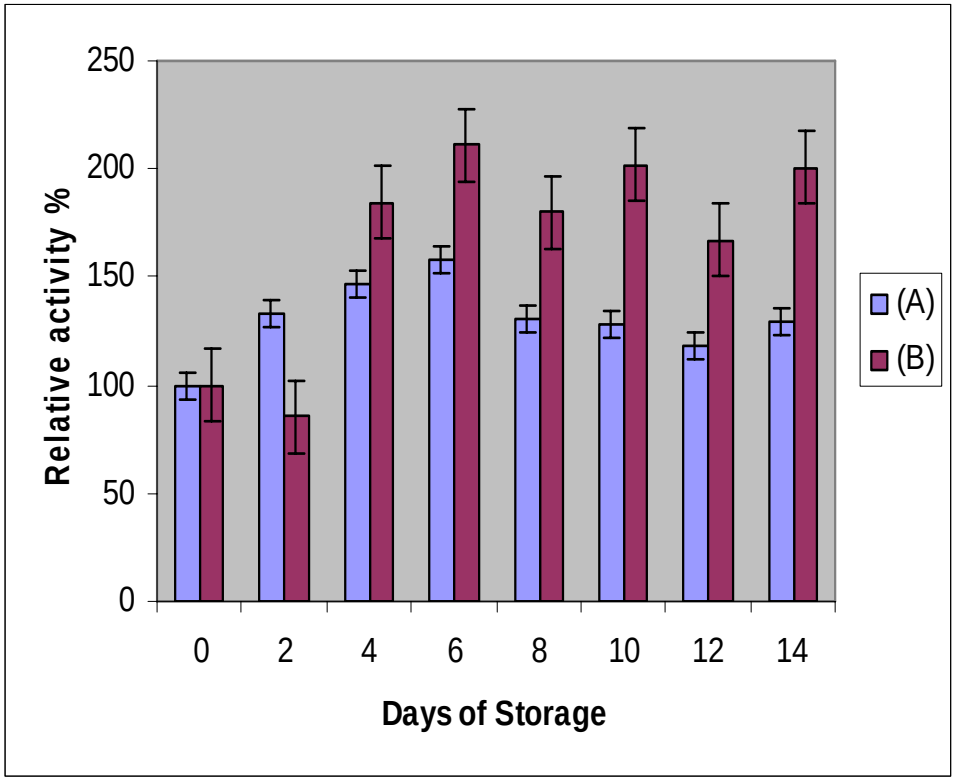


Figure 10. Effect of storage of fruits of *A. deliciosa* at 4°C and 22°C on superoxide dismutase (SOD) activity of crude extracts of whole fruit tissue. SOD activity of fresh fruits was considered as control and taken as 100%. SOD activity was determined in three different fruit extracts of whole tissue. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

3.6 Avoidance of Proteolytic Activity in Crude Extracts of *A. deliciosa* With Respect to SOD Activity

Kiwifruit contains a high level of proteolytic activity. Avoidance of proteolysis in crude extracts of *A. deliciosa* was investigated by adding a 'cocktail' of protease inhibitors to crude extract – the cocktail was added to appropriate volume of supernatant. It is known that some buffers accelerate the decomposition of protease inhibitors; therefore, protease inhibitors (PI) were also prepared by using kpi buffer (pH 7.8), to test the effect of buffer. Adding the 'cocktail' of PI resulted in 20% increase in SOD activity (Figure 11). There was no significant difference between 'cocktail' of PI prepared with water (PIW), and the cocktail prepared with buffer (PIB). Moreover, the samples using 'cocktail' of PI prepared with buffer had slightly higher SOD activity. Therefore the kpi buffer had no negative effect on PI function.

Another experiment was carried out to evaluate the effect of protease inhibitors individually. This is to test the influence of each component of the 'cocktail' on SOD activity. It was found that sample prepared with 'cocktail' lacking only iodoacetamide had 13% lower SOD activity than control (i.e. simple crude extract without any supplemented PI), and samples prepared with 'cocktails' of PI lacking only PMSF or 1,10 phenanthroline, had 32% and 25% higher activity than control, respectively (Figure 12). Among the protease inhibitors which were utilized, iodoacetamide had the most influence on SOD activity, since when the crude extract supplemented with the 'cocktail' lacking only iodoacetamide, SOD specific activity was slightly lower than control. Furthermore, presence of iodoacetamide in other crude extracts supplemented with 'cocktail' lacking another PI such as PMSF or 1,10 phenanthroline improved the SOD activity over that in the simple crude extract. Among the

protease inhibitors used, PMSF which is serine protease inhibitor, had the least influence on SOD activity in crude extract, since the absence of PMSF in the 'cocktail' still produced a significant increase in activity of the enzyme. The PI cocktail lacking 1,10 phenanthroline, which is a metallo-protease inhibitor, still had a major effect on SOD activity. Iodoacetamide inhibits the activity of cysteine proteases. One major cysteine protease present in kiwifruit is actinidin (E.C 3.4.22.14) (Podivinsky et al., 1992). The abundance of actinidin is probably linked to its influence on kiwifruit SOD activity. Overall, supplementing crude extracts with the 'cocktail' of PI improved the SOD activity in the crude extracts of the fruit, which might be due to selective inhibition of proteolytic activity (mainly actinidin) of kiwifruit. Further discussion regarding the influence of protease inhibitors on SOD activity during storage stability is provided in 3.7.1.

3.7 Characterization of SOD Activity in Crude Kiwifruit Extracts

SOD activity in the crude extracts of whole fruit of *A. deliciosa* was characterized. Storage stability, the effect of pH, thermo-stability, and SOD isosymes were investigated.

3.7.1. Storage Stability

Crude enzyme extracts prepared from whole fruit tissue were divided into two groups comprising: (A) extracts supplemented with the cocktail of synthetic protease inhibitors, and (B) simple crude extracts (without any PI supplemented). Aliquots of both groups of extracts were stored at room temperature (22°C) for seven days and their SOD activity was determined on daily basis. Another two sets of extracts from the same fruit were kept at 4°C and -20°C for a period of one month. Their SOD activity was measured at different time intervals. The activity of the stored samples was compared with that of the freshly prepared extracts.

Enzymes in crude extracts might be highly susceptible to both chemical and physical instabilities during storage, compared to their original intercellular environment. Chemical instability results in the generation of a new chemical entity by bond formation or cleavage. Physical instability involves changes in secondary and tertiary, or quaternary structure of the molecule, which can be manifested as denaturation, adsorption, aggregation, and precipitation. Both chemical and physical changes in proteins can result in loss of biological activity (Burgess, 1993).

Here, the chemical stability of SOD in crude extracts of *A. deliciosa* was investigated. The extract aliquots were stored and maintained in three different storage conditions; at room temperature for 7 days, at 4°C, and at -20°C, both for a period of 30 days. The crude extracts supplemented with a 'cocktail' of protease inhibitors were the samples that maintained a more stable SOD activity for up to 5 days when stored at room temperature (Table 4), although the activity found at the day 7, was about 50% (Figure 14). Samples without supplemented PI began to lose activity after 3 days of storage and lost 70% of their activity at the end of the 7-day storage period (Figure 13) which might be due to a better condition for proteolytic activity in these samples leading to faster degradation of the enzyme of interest. When the samples were stored at 4°C, the simple crude extracts (no PI supplemented) showed a decrease after 3 days of storage (Figure 15). Samples supplemented with PI also displayed the same pattern (Table 5), but there was an increase in their activity after 21 days of storage, before a decline in SOD activity by about 50% seven days later (Figure 16). It was found that simple crude extracts and crude extracts supplemented with PI retained their initial activity at the end of 30-day period stored at -20°C (Table 5). Furthermore, there was a significant difference after 30 days of storage between samples stored at 4°C and -20°C (Figures 15&16).

During the storage stability study of SOD in crude extracts of *A. deliciosa*, the influence of synthetic protease inhibitors added to the crude extract on SOD activity was observed. The SOD activity, at least to some extent, was stabilized by using the 'cocktail' of PI.

Supplementing crude extracts with protease inhibitors, was also found to be effective on samples stored at 4°C, by stabilizing the enzyme activity for a longer period of time. The samples retained their activity for about 80% after

21 days of storage. However, there was a loss in activity after 3rd day, and remained constant by 14th day, which might be due to possible interference of other antioxidants and proteins, and interactions between SOD and the chemical compounds used. It was found that supplementing PI to samples stored at -20°C, was not required, at least during the course of this experiment, since simple crude extracts retained their initial activity by 30th day, which might be due lack of proteolytic activity at low temperatures.

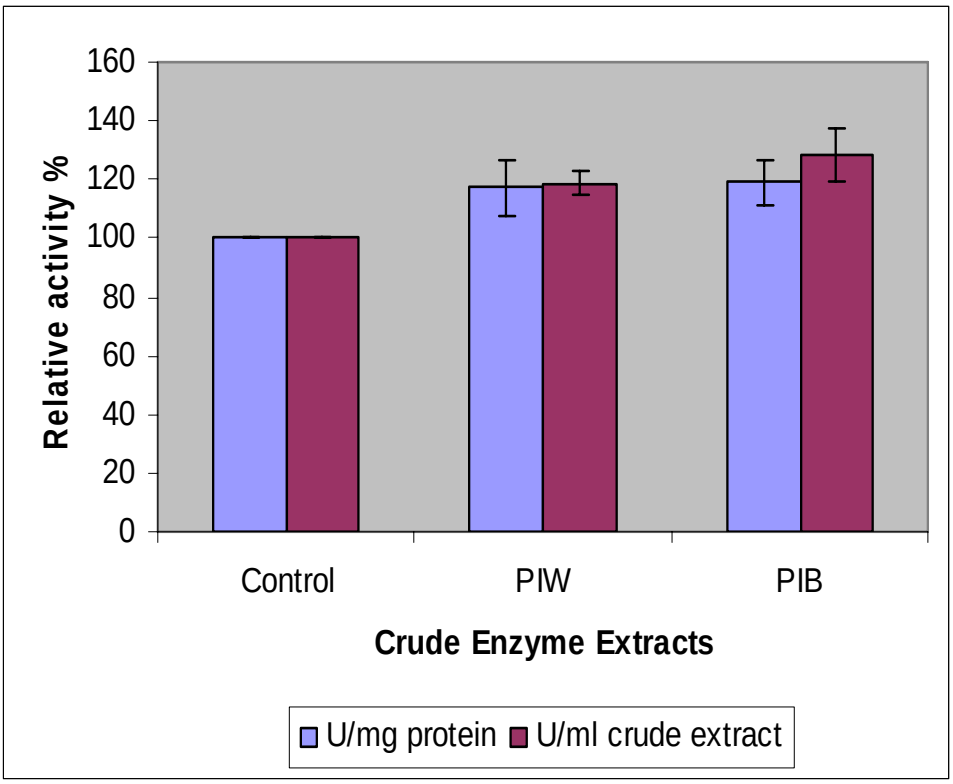


Figure 11. Effect of protease inhibitors on superoxide dismutase (SOD) activity in crude extracts of whole fruit of *A. deliciosa*. PIW and PIB, represent the 'cocktail' of protease inhibitors (PI) prepared with water, and prepared with kpi buffer (7.8), respectively. Crude extract without added cocktail of protease inhibitors was considered as control and taken as 100%. SOD activity was determined in three different fruit extracts. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

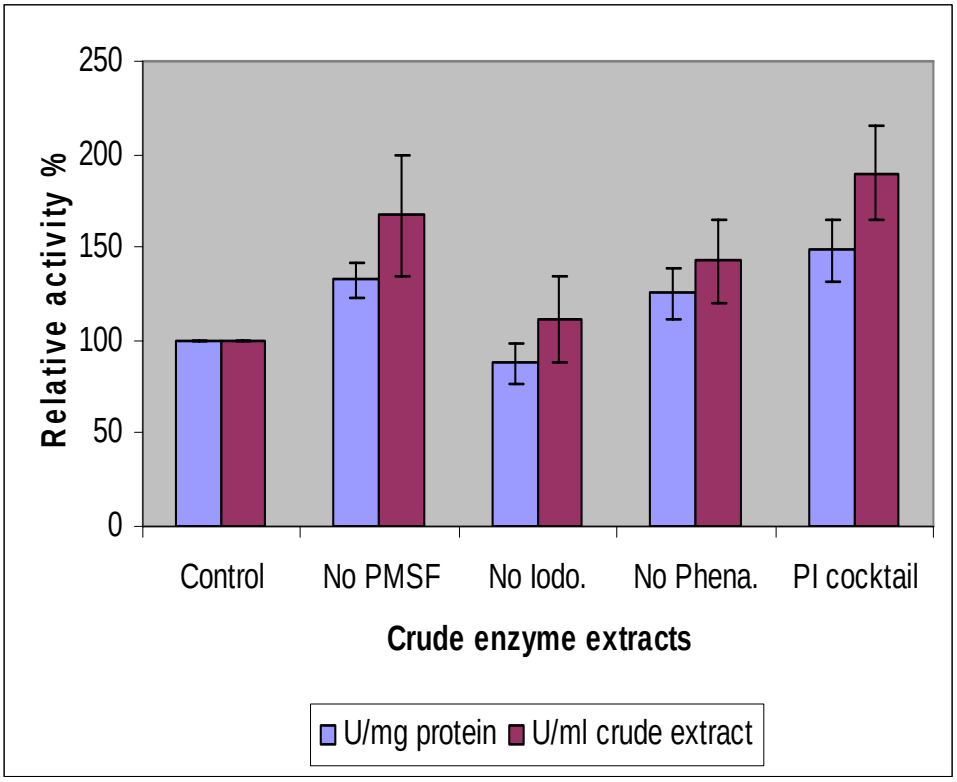


Figure 12. Individual effect of protease inhibitors on superoxide dismutase (SOD) activity in crude extracts of whole fruit of *A. deliciosa*. SOD activity in crude extract without any added protease inhibitors was considered as control and taken as 100%. *No PMSF*; represents the crude extracts supplemented with the 'cocktail' of PI lacking PMSF, *No Iodo*.; represents the crude extracts supplemented with the 'cocktail' of PI lacking iodoacetamide, *No Phena*.; represents the crude extracts supplemented with the 'cocktail' of PI lacking 1,10 phenanthroline, *PI cocktail*; represents the crude extracts supplemented with the 'cocktail' of all PI. SOD activity was determined in three different fruit extracts of whole tissue. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

Table 4. Total SOD activity in crude extracts during the assessment of enzyme storage stability at room temperature**.**

Storage Condition	Type of Crude Extract	Days of Storage							
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Room Temperature	Simple crude extract	93	108	118	82	74	50	31	27
	Crude extract + cocktail of protease inhibitors	222	170	274	159	288	283	182	113

Table 5. Total SOD activity in crude extracts during the assessment of enzyme storage stability at 4°C and -20°C.

Storage Condition	Type of Crude Extract	Days of Storage					
		0	**3**	**7**	**14**	**21**	**30**
4°C							
	Simple crude extract	93	140	92	61	52	34
	Crude extract + cocktail of protease inhibitors	222	276	132	128	197	127
-20°C							
	Simple crude extract	93	142	133	228	228	111
	Crude extract + cocktail of protease inhibitors	222	196	192	182	315	190

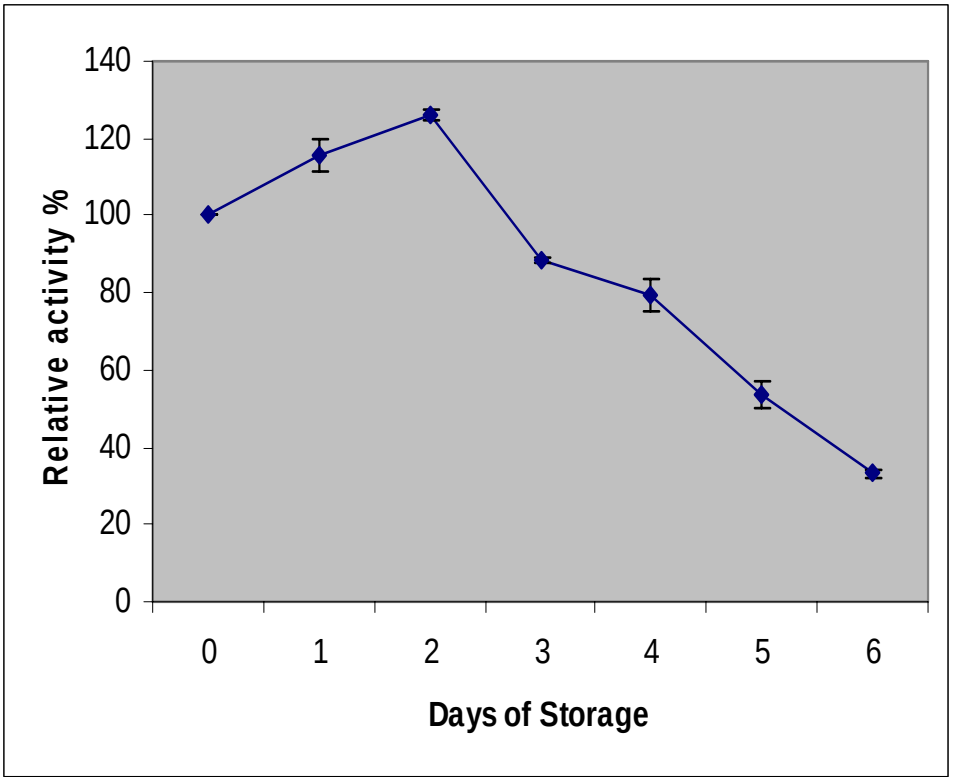


Figure 13. Effect of storage at room temperature (22°C) on superoxide dismutase (SOD) specific activity of simple crude extracts of whole fruit tissue of *A. deliciosa*. The SOD activity in fresh extracts was considered as control and taken as 100%. SOD activity was determined in three different fruit extracts. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

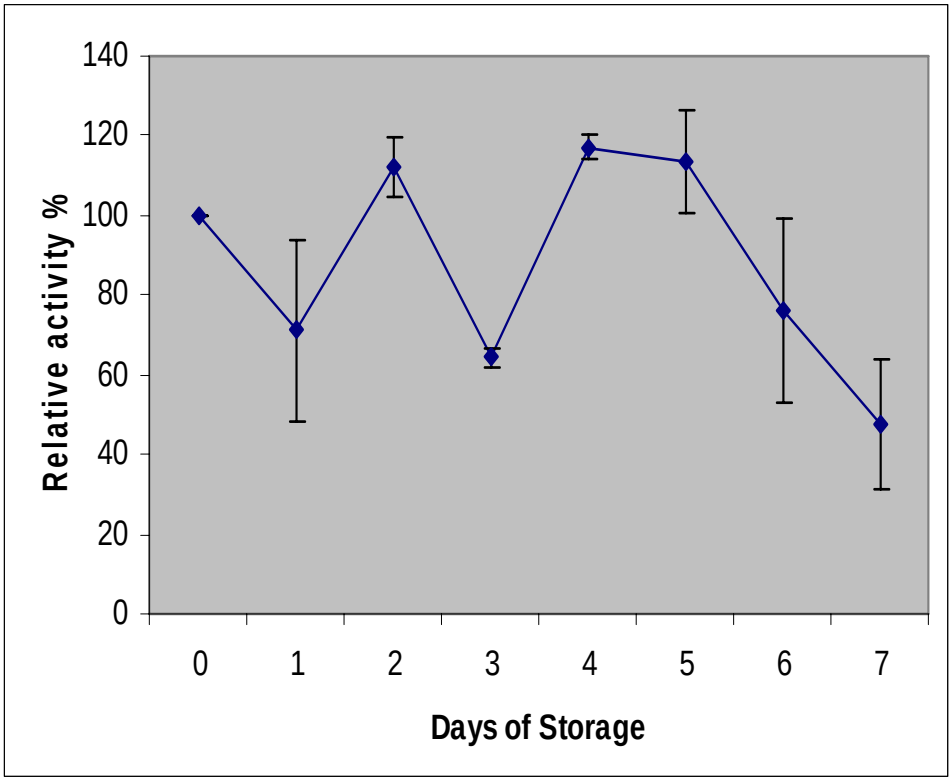


Figure 14. Effect of storage at room temperature (22°C) on superoxide dismutase (SOD) specific activity of crude extracts of whole fruit tissue of *A. deliciosa* supplemented with the cocktail of protease inhibitors. The SOD activity in fresh extracts was considered as control and taken as 100%. SOD activity was determined in three different fruit extracts. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

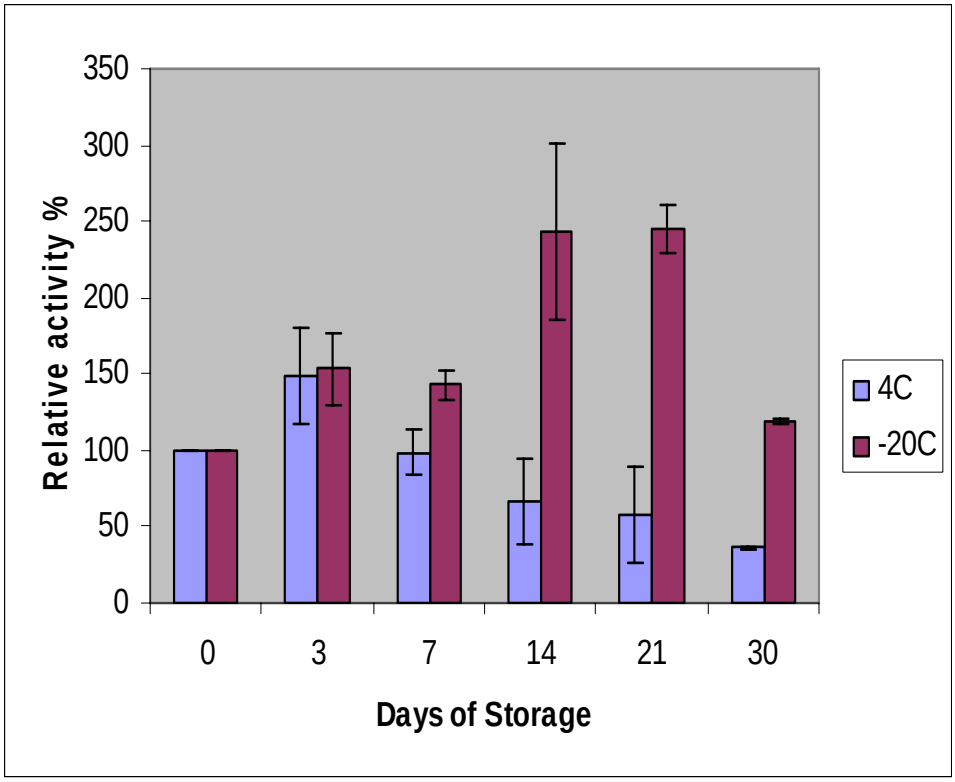


Figure 15. Effect of storage at 4°C and -20°C on superoxide dismutase (SOD) specific activity in simple crude extracts (without the added 'cocktail' of PI) of whole fruit tissue of *A. deliciosa*. SOD activity was determined in three different fruit extracts. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

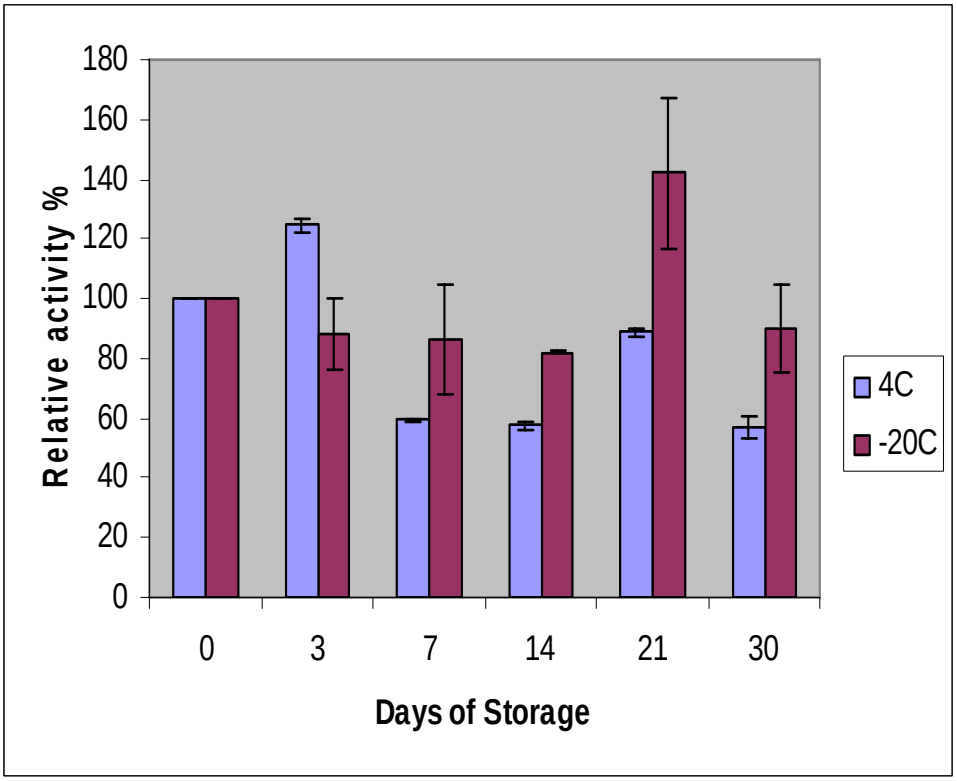


Figure 16. Effect of storage at 4°C and -20°C on superoxide dismutase (SOD) specific activity of crude extracts of whole fruit tissue of *A. deliciosa* supplemented with the 'cocktail' of PI. SOD activity was determined in three different fruit extracts. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

3.7.2 Thermal Stability

The SOD in enzyme crude extracts was stable at 100°C for 1.5 minutes. The enzyme activity was decreased significantly to 60% of that without boiling after 3 min of boiling. After heating for 20 min, the loss of crude extract SOD activity was almost complete (Figure 17). Increasing SOD activity after 1.5 min of boiling might be due to inactivation of peroxidases which could interfere with SOD activity assay (Giannopolitis and Ries, 1977).

3.7.3 The effect of pH on SOD activity

The pH activity profile of SOD in crude extracts of *A. deliciosa* was studied at standard assay conditions by using three different buffer systems in three different pH ranges. Maximum SOD activity was observed at pH 7.8. The pH curve was characterized by a rapid decrease in enzyme activity above pH 7.8 and below pH 7.2. The resultant graph confirms that the chosen assay condition was the optimum condition (pH 7.8) (Figure 18).

3.7.4 Estimation of pH stability

Activity profiles of SOD in crude extracts of A. deliciosa after have been incubated at 25°C and at different pH values (between 2.6 and 10). According to the resultant graph, optimum SOD activity was found in the buffer at pH 7.2. The enzyme retained more than 90% and 60% of its activity after incubation at pH 7.8 and pH 6.0, respectively. SOD in crude extract of *A. deliciosa* was inactivated by nearly 60% at pH 2.6-5.0, and retained almost 50% of its activity at pH 8.5 till 10. Thus, it is suggested that the overall dismutation rate of SOD in *A. deliciosa* crude extract was dependent on pH in the range of 7.2-7.8 (Figure 19).

3.7.5 Determination of Isozymes

3.7.5.1 Gel Electrophoresis

Discontinuous gel electrophoresis of crude extracts was performed with 12% separating (pH 8.8) and 4% stacking gels at room temperature at 200 volts for 42 minutes. Crude extracts of whole fruit tissue and seed were utilized. The gel was prepared in the Mini protein gel system (BioRad, California, USA). Gel electrophoresis yielded weak and diffuse bands in a faintly stained background (Plate 9a). Several attempts were carried out to tackle the problem including supplementing the 'cocktail' of PI; adding azide to the crude extract to inhibit the possible interference of peroxidases; and partially purification the enzyme by ammonium sulphate precipitation (as described next in 3.7.5.2), which were all unsuccessful. Even implementing the gel electrophoresis steps using ice cold running buffer (Tris-Gly, pH 8.3, 25 mM) and placing the electrophoresis apparatus in icy water which was reported to prevent the inhibition of SOD due to warming the gel because of high voltage (Burke and Oliver, 1992) was not successful. This might be due to the presence of phenolics compounds or peroxidases that mimic the SOD activity. In another attempt, crude extracts of whole fruit and seed were analyzed using a Mini IEF (isoelectric focusing) gel electrophoresis apparatus (BioRad, California, USA) to detect the SOD isoforms (Plate 9b). With this gel technique some distinct bands were visible. One for fruit specific and some seed specific bands. This is promising but still needs further improvement.

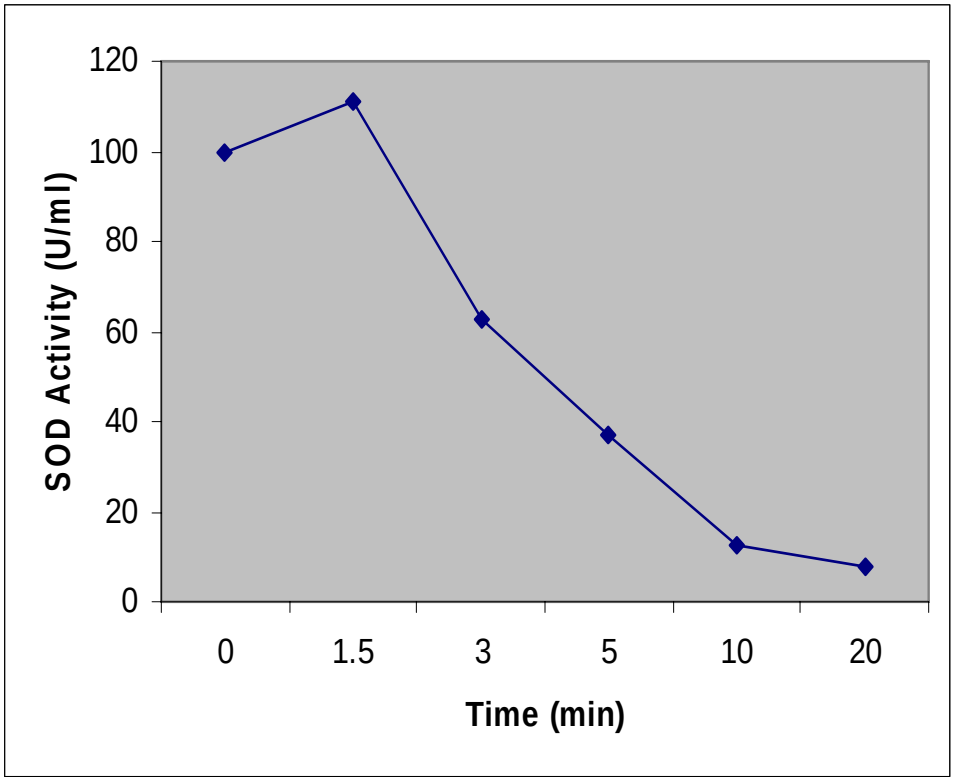


Figure 17. Thermal stability of superoxide dismutase in crude extracts of whole fruit of *A. deliciosa*. The enzyme activity of un-boiled extracts was considered as control and taken as 100%. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

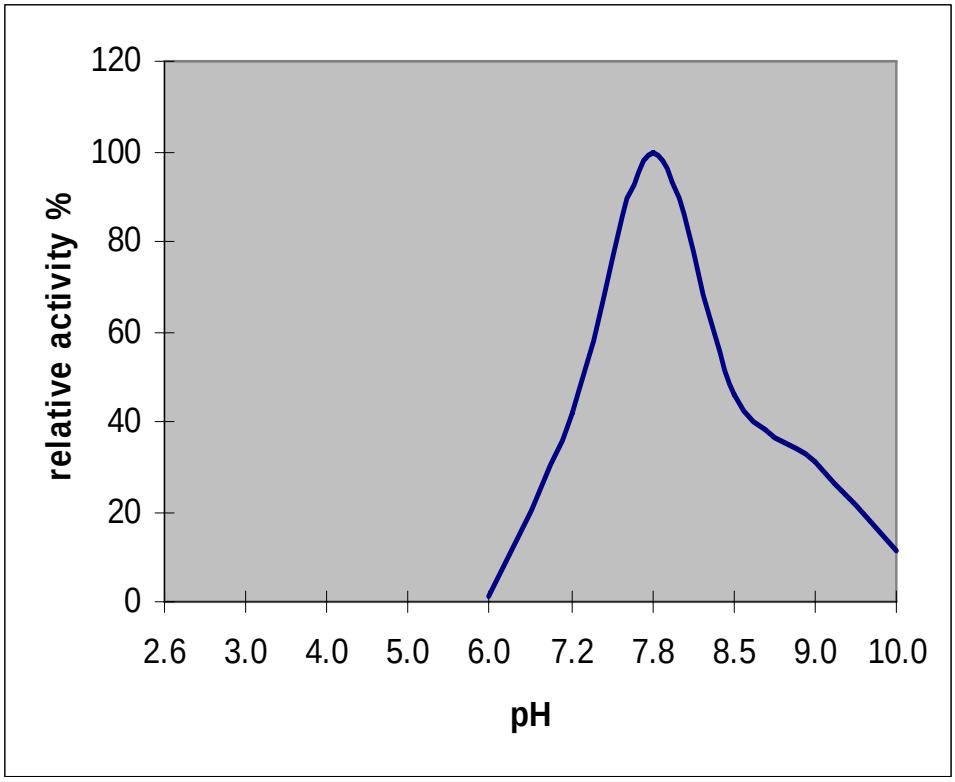


Figure 18. The effect of pH in the assay reaction mixture on superoxide dismutase activity of crude enzyme extracts of whole fruit of *A. deliciosa*. Activity of enzyme at pH 7.8 was considered as 100%. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

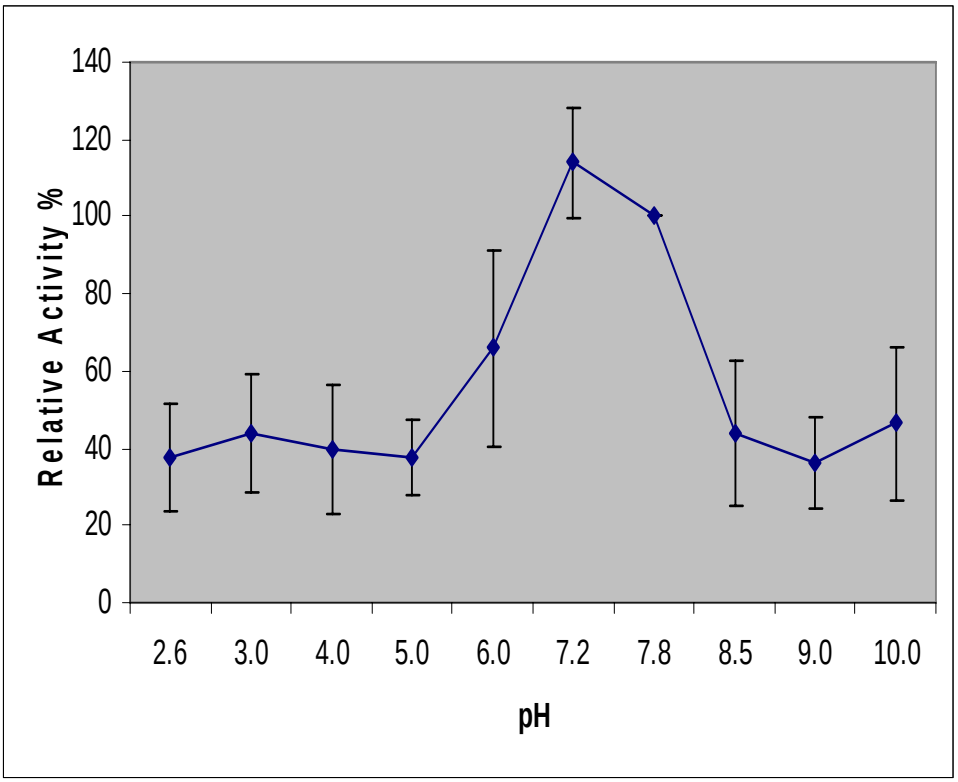


Figure 19. The effect of pH on stability of superoxide dismutase of crude enzyme extracts of whole fruit of *A. deliciosa*. Stability of enzyme in pH 7.8 was considered as 100%. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

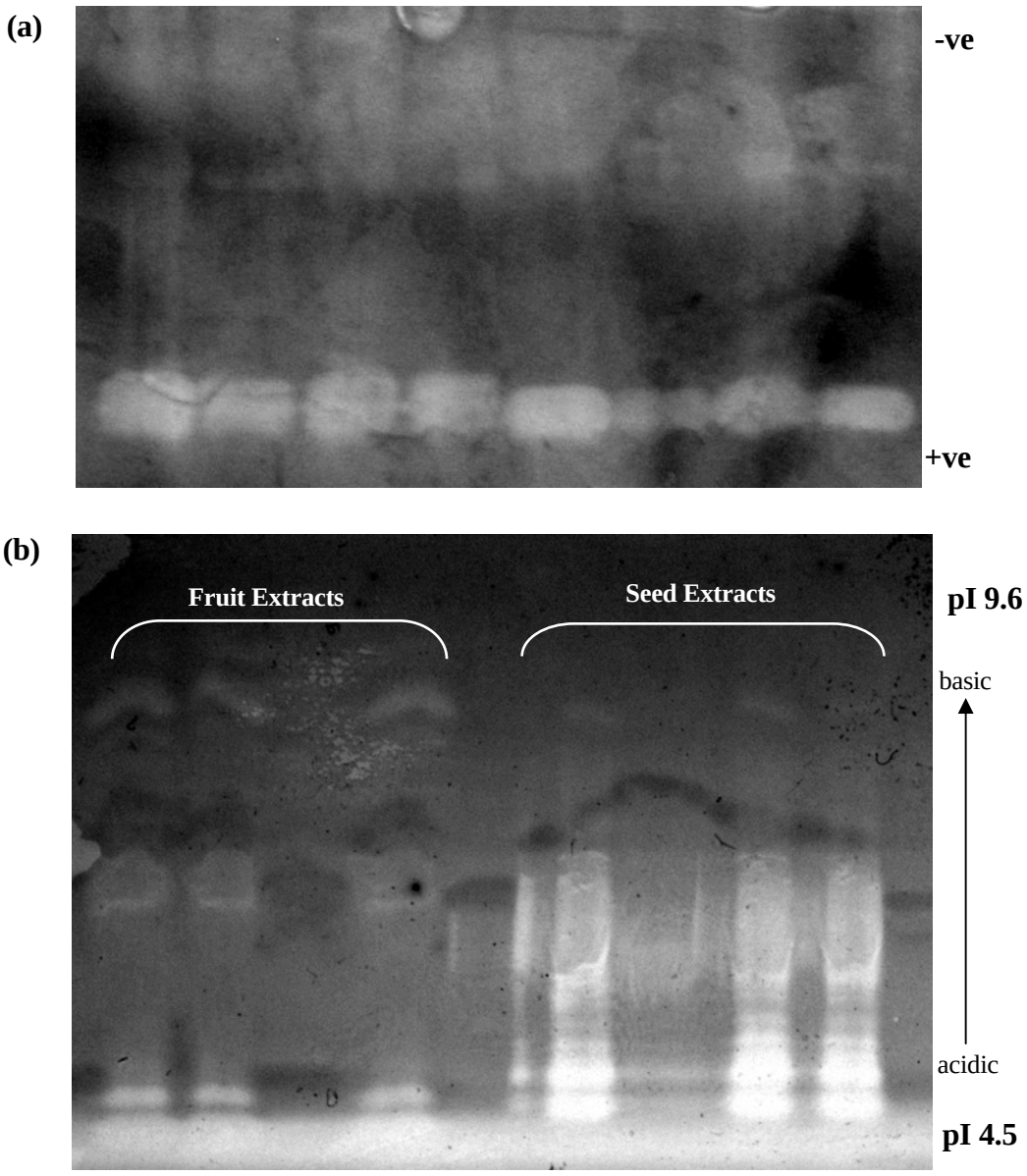


Plate 9. Electrophoresis analysis. (**a)** Activity staining of SODs after native-PAGE. **(b)** Activity staining of SODs on IEF gel

3.7.5.2 Purification by Ammonium Sulphate Precipitation

Ammonium sulphate $(NH_4)_2SO_4$ was utilized to achieve the bulk purification of enzyme crude extract. Here, the method was optimized by two major steps; the first was to optimize the best ratio to remove the unwanted proteins. 20, 25, 30, 35, 40, 45, 50, and 60% (w/v) saturation with ammonium sulphate were used and after centrifugation at 10000×g for 20min, the precipitate was removed. SOD activity and protein content in the supernatant were determined. As can be seen from Figure 20a, the highest SOD activity was 19.53 $U.mg^{-1}$ obtained with 40% ammonium sulphate precipitation, which was the best concentration of the salt to remove the unwanted proteins. The same experiments were repeated to optimize the second step of ammonium sulphate precipitation, to collect the highest level of SOD actvity from crude extract. Experiments were started from 40% (w/v) to a series of ratios, 45, 50, 55, 60, 65, 70, 75, 80, and 90% (w/v) saturation with ammonium sulphate. After centrifugation at 10000×g for 20min, the precipitate was dissolved in the initial volume of 0.1 M phosphate buffer, pH 7.8. SOD activity and protein content were determined. As can be seen from Figure 20b, the highest SOD activity was obtained from 60% (w/v) saturation with ammonium sulphate, which was 20.95 $U.mg^{-1}$. The specific activity, % of yield and the purity factor at each step of purification are summarized in Table 6. The information obtained here should be a useful starting point for further projects aiming to isolate different SOD isozymes in kiwifruit.

3.8 The Influence of Kiwifruit Cell Wall Autolysis on SOD Activity of Fruit Tissues

It is known that changes in the firmness of fruit tissues involves alterations to the structure of cell walls leading to release of intercellular enzymes and proteins to extracellular spaces resulting in changes of enzymic. In order to demonstrate the possible influence of cell wall lysis during kiwifruit ripening (post-harvest) on SOD activities in fruit tissues, an experiment was carried out by comparing relatively firm (type A) and soft (type B) fruits (Figure 21). There was a significant difference in SOD activity of different tissues in relatively firm fruits. Inner pericarp had the highest SOD activity, followed by core, and outer pericarp. Interestingly, results showed that unlike relatively firm fruits, there was no significant difference in SOD activity among different tissues tested in soft fruits. Of the group of fruits compared, SOD activity of outer pericarp showed the greatest difference, followed by inner pericarp and core. Decompartmentalization of enzymes and proteins including SOD due to softening, might be the main factor affecting the changes in SOD/ $O_2^{\cdot -}$ ratio.

3.9 Determination of Presence of Endogenous Inhibitors and Promoters in Different Parts of Fruit of *A. deliciosa*

The presence of endogenous inhibitors was tested by mixing enzyme extract from one tissue of fruit with the same volume of extract from another tissue of the same fruit and comparing the enzyme activity of the mixture with that of an expected value (Table 6). Results showed that 31-69% inhibition of activity of mixed extracts. It would be interesting to investigate the nature of this apparent inhibition of SOD activity in future studies.

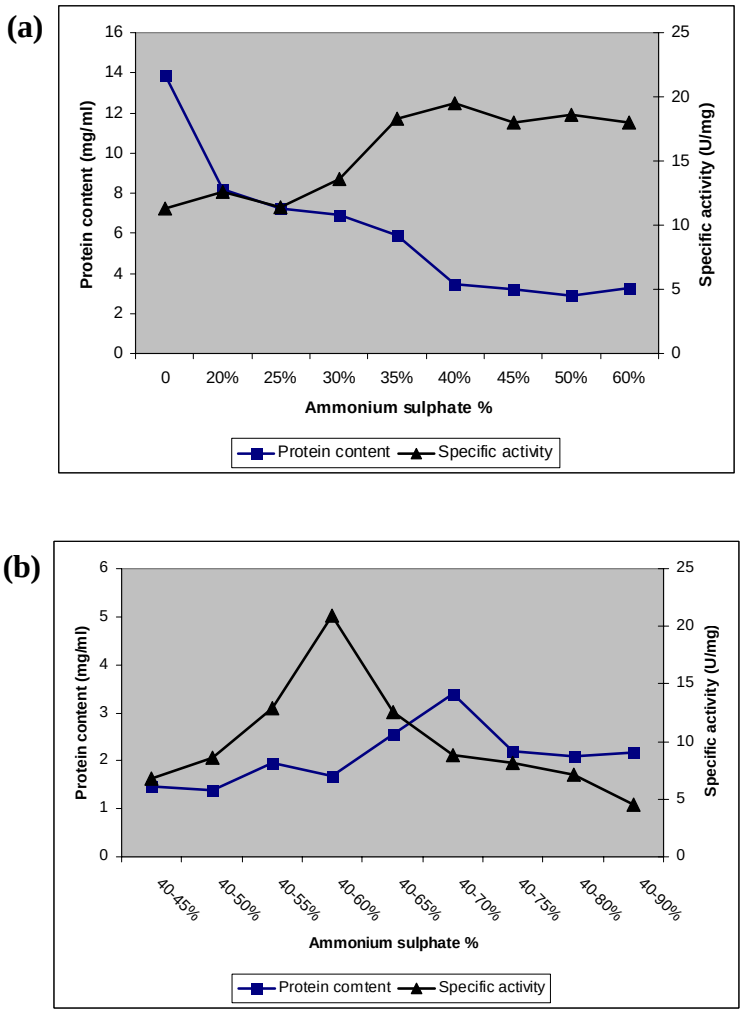


Figure 20. Variation of specific activity and protein content with different ammonium sulphate%. (a) First step of optimization of protein precipitation by ammonium sulphate. (b) Second step of optimization of protein precipitation by ammonium sulphate.

Table 6 Purification of SOD activity in kiwifruit crude extracts

Steps	Activity (U/ml)	Total activity (U)	Protein content (mg/ml)	Specicfic activity (U/mg)	Volume (ml)	Yield (%)	Purification fold
Crude	31	627.45	9.2	3.36	20	100	1
40-60%	70	105.15	3.34	20.95	1.5	16.75	6.23

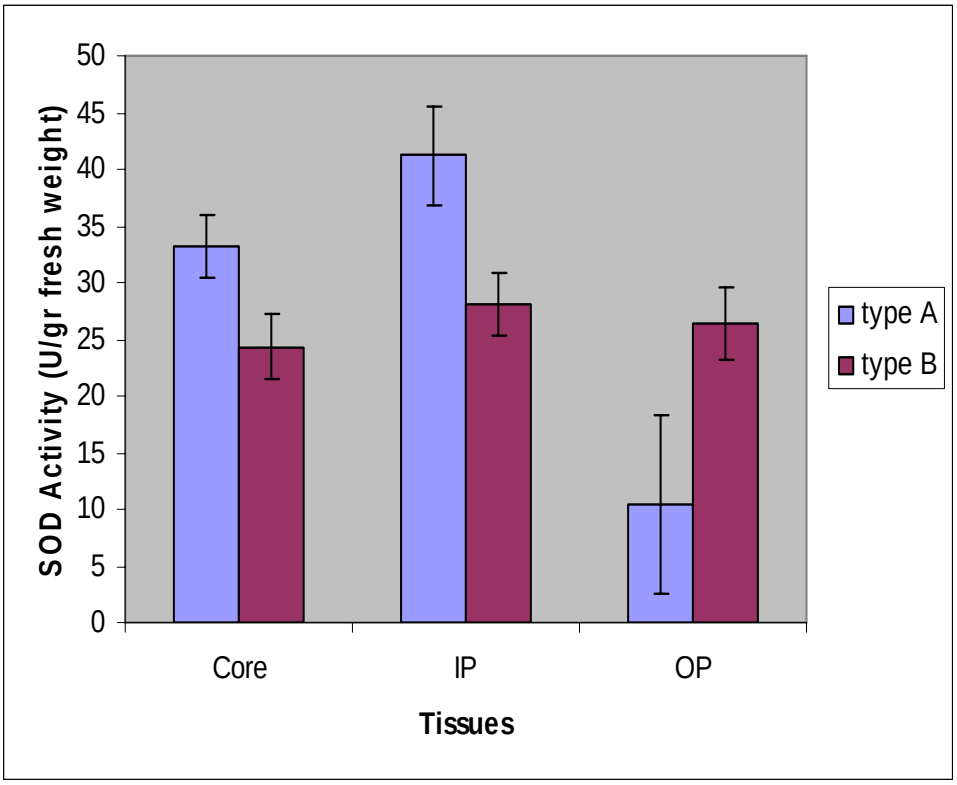


Figure 21. SOD activity in crude extracts of three different tissues of fruit of *A. deliciosa*. Type A and type B represent the relatively firm and soft fruits, respectively. SOD activity was determined in three different extracts of each tissue. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

Table 7. Effect of mixing enzyme extracts from two different tissues of *A. deliciosa* on Superoxide dismutase activity (% inhibition) *

Mixture of two different parts	% inhibition	SE
Seed – Core	**53**	**4.67**
Seed – Locule	**48**	**3.97**
Seed – Outer Pericarp	**31**	**2.95**
Core – Locule	**55**	**5.52**
Core – Outer Pericarp	**49**	**3.85**
Locule – Outer Pericarp	**69**	**6.57**

***** Superoxide activity (% inhibition) was determined in tissue extracts from four different tissues. Three replicate assays were carried out with each extract. Mean values ± standard errors (SE) are presented.

CHAPTER IV

Concluding Remarks

The high participation of fruits and vegetables in the human diet because of their ability to neutralize active oxygen species, hazardous for health, is of utmost importance. Plant tissue antioxidant capacity is closely associated with activity of "free radical scavenging enzymes" such as superoxide dismutase (SOD). It is now widely accepted that SODs are able to weaken, or eliminate altogether, a wide range of toxic effects produced by the exposure to $O_2^{\cdot -}$ generating systems. The present work established the activity of superoxide dismutase in three species of *Actinidia*. Among the three species tested *A. deliciosa* showed slightly higher activity, although the difference among species with regard to SOD activity was not significant. Due to time constraints and lack of access to fruits growing on vines, experiments were carried out on post-harvest kiwifruit and the main focus of this study was on

A. deliciosa. Therefore the exact period for which the fruits had been on display before being used in this study was not known.

Kiwifruit are harvested mature and unripe, and considerable fruit softening must take place before fruits can be eaten. Several studies reported large changes in a number of physiological and chemical changes not only in whole fruit but also in different tissue types within the fruit (Lallu et al., 1989). Here, it was found that among the fruit tissues, seed contained the highest SOD activity. Besides, study of storage stability demonstrated that, cold temperature could induce the activity of superoxide dismutase during ripening, suggesting that more ripe and relatively softer fruits have higher nutritional value with regard to SOD activity.

Investigations regarding the detection of $O_2^{\cdot -}$ in fruit tissues confirmed the presence of SOD and its vital role against the toxicity of superoxide anion. Study of SOD isozymes did not produce clear results, despite several different attempts which were implemented to tackle the problem. The presence of phenolics and other antioxidants that might mimic the SOD function could be the source of erroneous results. The mini IEF method was promising, but further improvements are still needed.

The overall findings suggest that kiwifruit could be used as a good source of SOD and the long storage life of kiwifruit could be an advantage with respect to inducible characteristic of the enzyme due to cold storage.

Directions for future studies

Further studies into the biochemical/molecular mechanism(s) responsible for the apparent increase in SOD activity when kiwifruit are stored at 4°C should open a new avenue of kiwifruit biochemistry research.

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